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(57) Abstract

A reshaped human antibody or reshaped human antibody fragment having specificity for human polymorphic epithelial mucin (PEM) is produced by transferring the complementarity determining regions (CDRs) from a murine anti-HMFG hybridoma cell line HMFG1 into a human antibody variable region framework. The reshaped molecule can be used in the treatment or diagnosis of cancer.

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SPECIFIC BINDING AGENTS

This invention relates to specific binding agents, and in particular to polypeptides containing amino acid sequences that bind specifically to other proteinaceous or non-proteinaceous materials. The invention most particularly concerns the production of such specific binding agents by genetic engineering.

Antibody structure

Natural antibody molecules consist of two identical heavy-chain and two identical light-chain polypeptides, which are covalently linked by disulphide bonds. Figure 14 of the accompanying drawings diagramatically represents the typical structure of an antibody of the IgG class. Each of the chains is folded into several discrete domains. The N-terminal domains of all the chains are variable in sequence and therefore called the variable regions (V-regions). The V-regions of one heavy (VH) and one light chain (VL) associate to form the antigen-binding site. The module formed by the combined VH and VL domains is referred to as the Fv (variable fragment) of the

antibody. The C-terminal ends of both heavy and light chains are more conserved in sequence and therefore referred to as the constant regions. Heavy chain constant regions are composed of several domains, eg. the heavy chain constant region of the gamma-isotype (IgG) consists of three domains (CH1, CH2, CH3) and a hinge region which connects the CH1 and CH2 domains. The hinges of the two heavy chains are covalently linked together by disulphide bridges. Light chains have one constant domain which packs against the CH1 domain. The constant regions of the antibody molecule are involved in effector functions such as complement lysis and clearing by Antibody Dependant Cell Cytotoxicity (ADCC). Classical digestion of an antibody with the protease papain yields three fragments. One fragment contains the CH2 and CH3 domains and, as it crystallises easily, was called the Fc fragment. The other two fragments were designated the Fab (antigen-binding) fragments, they are identical and contain the entire light chain combined with the VH and CH1 domain. When using pepsin, the proteolytic cleavage is such that the two Fabs remain connected via the hinge and form the $(Fab)_2$ fragment. Each of the domains is represented by a separate exon at the genetic level.

The variable regions themselves each contain 3 clusters of hypervariable residues, in a framework of more conserved sequences. These hypervariable regions interact with the antigen, and are called the Complementarity Determining Regions (CDRs). The more conserved sequences are called the Framework Regions (FRs). See Kabat et al (1987). X-ray studies of antibodies have shown that the CDRs form loops which protrude from the top of the molecule, whilst the FRs provide a structural beta-sheet framework.

Modified antibodies

In one embodiment, the invention relates to so-called "reshaped" or "altered" human antibodies, ie. immunoglobulins having essentially human constant and framework regions but in which the complementarity determining regions (CDRs) correspond to those found in a non-human immunoglobulin, and also to corresponding reshaped antibody fragments.

The general principles by which such reshaped human antibodies and fragments may be produced are now well-known, and reference can be made to Jones et al (1986), Riechmann et al (1988), Verhoeyen et al (1988), and EP-A-239400 (Winter). A comprehensive list of relevant literature references is provided later in this specification.

Reshaped human antibodies and fragments have particular utility in the in-vivo diagnosis and treatment of human ailments because the essentially human proteins are less likely to induce undesirable adverse reactions when they are administered to a human patient, and the desired specificity conferred by the CDRs can be raised in a host animal, such as a mouse, from which antibodies of selected specificity can be obtained more readily. The variable region genes can be cloned from the non-human antibody, and the CDRs grafted into a human variable-region framework by genetic engineering techniques to provide the reshaped human antibody or fragment. To achieve this desirable result, it is necessary to identify and sequence at least the CDRs in the selected non-human antibody, and preferably the whole non-human variable region sequence, to allow

identification of potentially important CDR-framework interactions.

Antibodies raised against the human milk fat globule (HMFG), generally in a delipidated state, can exhibit a broad spectrum of reactivity with epithelial origin neoplasms, particularly carcinomas of the breast, ovary, uterus and lung. See Taylor-Papadimitriou et al (1981) and Arklie et al (1981). One well-characterised antibody (designated HMFG1) is known to bind to a component of the HMFG, also found in some body tissues, some cancer tissues and urine, which has been designated polymorphic epithelial mucin (PEM) (Gendler et al, 1988). Binding is thought to involve the peptide core of the PEM. Corresponding useful specificity can be achieved by raising antibodies against cancer cells, for example breast cancer cell lines.

EP-A2-0369816 (The University of Melbourne, Xing et al) describes monoclonal antibodies specific for human polymorphic epithelial mucin, which bind to a defined amino acid sequence. It is suggested in EP-A2-0369816 that the described antibodies may be "humanised" according to the method of Riechmann et al (1988). However, Xing et al do not describe the actual preparation of any such reshaped anti-PEM antibodies.

Summary of the invention

The invention provides, as one embodiment, a synthetic specific binding polypeptide having specificity for a polymorphic epithelial mucin (PEM), and especially a synthetic specific binding polypeptide having anti-human milk fat globule (HMFG) specificity, containing one or more of the CDRs depicted in Figures 1 and 2 of the

accompanying drawings. By synthetic, we particularly mean that the polypeptide is produced by recombinant DNA technology, and to that extent at least is different from a naturally-occurring or naturally-induced specific binding agent having identical specificity. Alternatively, the synthetic polypeptide has been produced by artificially assembling a sequence of amino acids to produce a novel or nature-identical molecule. The synthetic polypeptide can be equivalent to an intact conventional antibody, or equivalent to a multiple or single-chain fragment of such an antibody, or can be simply a material that includes one or more sequences that confer the desired specific binding capability.

The invention provides as an important embodiment a reshaped human antibody, or a reshaped human antibody fragment, having anti-PEM specificity, and especially having anti-HMFG specificity, containing one or more of the CDRs depicted in Figures 1 and 2 of the accompanying drawings. Preferably, the reshaped antibody or fragment of the invention contains all 3 of the CDRs depicted in Figure 1 of the accompanying drawings, in a human heavy chain variable region framework. Alternatively, or in addition, the reshaped antibody or fragment of the invention contains all 3 of the CDRs depicted in Figure 2 of the accompanying drawings, in a human light chain variable region framework.

Another embodiment of the invention is a reshaped antibody or reshaped antibody fragment containing a protein sequence as depicted in Figure 12 and/or Figure 13 of the accompanying drawings.

Other important embodiments of the invention are an expression vector incorporating a DNA sequence as depicted

in Figure 12 and/or Figure 13 of the accompanying drawings, and an expression vector incorporating a DNA sequence encoding one or more of the protein sequences designated as being a CDR in Figure 1 and/or Figure 2 of the accompanying drawings.

An important aspect of the invention is a stable host cell line containing a foreign gene that causes the host cell line to produce a specific binding agent according to the invention. This can be a stable host cell line containing a foreign gene that encodes at least one of the amino acid sequences designated as being a CDR in Figure 1 and/or Figure 2 of the accompanying drawings, together with a protein framework that enables the encoded amino acid sequence when expressed to function as a CDR having specificity for HMFG.

The invention also provides an immortalised mammalian cell line, or a yeast, or other eukaryotic cell, or a prokaryotic cell such as a bacterium, producing a reshaped antibody or fragment according to the invention.

Another important aspect of the invention is a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, having specificity equivalent to that of the gamma-1, kappa anti-HMFG monoclonal antibody "HMFG1".

The invention also provides two novel plasmids, pSVgpt-HuVHHMFG1-HuIgG1 and pSVneo-HuVkHMFG1-HuCk, and these plasmids can be used in the production of a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment.

These plasmids are contained in novel E.coli strains NCTC 12411 and NCTC 12412, respectively.

Other aspects of the invention are:

a) A DNA sequence encoding a reshaped human antibody heavy-chain variable region having specificity for HMFG, as contained in E.coli NCTC 12411.

b) A DNA sequence encoding a reshaped human antibody light-chain variable region having specificity for HMFG, as contained in E.coli NCTC 12412.

c) A reshaped human antibody heavy-chain variable region having specificity for HMFG, producible by means of the expression vector contained in E.coli NCTC 12411.

d) A reshaped human antibody light-chain variable region having specificity for HMFG, producible by means of the expression vector contained in E.coli NCTC 12412.

e) A reshaped human antibody or reshaped human antibody fragment, comprising at least one variable region according to c) or d) above.

A particular embodiment of the invention is therefore a reshaped human antibody or reshaped human antibody fragment possessing anti-HMFG specificity and incorporating a combination of CDRs (which may, for example, be cloned from a murine anti-HMFG immunoglobulin) having the amino acid sequences identified as CDR1, CDR2 and CDR3 respectively in Figures 1 and 2 of the accompanying drawings, which respectively represent the heavy chain variable region (VH) and light chain variable region (Vk) of a murine anti-HMFG monoclonal antibody that

we have cloned and sequenced. In the case of an intact antibody, or a fragment comprising at least one heavy chain variable region and at least one light chain variable region, the reshaped antibody or fragment preferably contains all six CDRs from the non-human source. To be most effective in binding, the CDRs should preferably be sited relative to one another in the same arrangement as occurs in the original non-human antibody, e.g. the VH CDRs should be in a human VH framework, and in the order in which they occur naturally in the non-human antibody.

As will be apparent to those skilled in the art, the CDR sequences and the surrounding framework sequences can be subject to modifications and variations without the essential specific binding capability being significantly reduced. Such modifications and variations can be present either at the genetic level or in the amino acid sequence, or both. Accordingly, the invention encompasses synthetic (reshaped) antibodies and fragments that are functionally equivalent to those described herein having precisely defined genetic or amino acid sequences.

The invention can also be applied in the production of bi-specific antibodies, having two Fab portions of different specificity, wherein one of the specificities is conferred by a reshaped human variable chain region incorporating one or more of the CDRs depicted in Figures 1 and 2 of the accompanying drawings.

The invention can also be applied in the production of so-called single-chain antibodies (for example, as disclosed in Genex EP-A-281604), and also to polysaccharide-linked antibodies (see Hybritech EP-A-315456), and other modified antibodies.

Any human constant regions (for example, gamma 1, 2, 3 or 4-type) can be used.

Antibody fragments retaining useful specific binding properties can be $(Fab)_2$, Fab, Fv, VH or Vk fragments. These can be derived from an intact reshaped antibody, for example by protease digestion, or produced as such by genetic engineering.

Practical applications of the invention

An important aspect of the invention is a reshaped human anti-HMFG antibody or fragment, as defined above, linked to or incorporating an agent capable of retarding or terminating the growth of cancerous cells, or to an imaging agent capable of being detected while inside the human body. The invention also includes injectable compositions comprising either of such combinations in a pharmaceutically acceptable carrier, such as saline solution, plasma extender or liposomes. The invention also includes the use, in a method of human cancer therapy or imaging, of a reshaped human anti-HMFG antibody or fragment as defined above. The invention further includes the use of such an antibody or fragment for the manufacture of a medicament for therapeutic application in the relief of cancer in humans, or the use of such an antibody or fragment in the manufacture of a diagnostic composition for in-vivo diagnostic application in humans.

The Fc region of the antibody, itself using pathways and mechanisms available in the body, such as complement lysis and antibody dependent cellular cytotoxicity, can be used to affect adversely the growth of cancerous cells. In this embodiment, no additional reagent need be linked to the reshaped antibody.

Examples of agents capable of affecting adversely the growth of cancerous cells include radioisotopes, such as Yttrium 90 and Iodine 131; drugs such as methotrexate; toxins such as ricin or parts thereof; and enzymes which may for example turn an inactive drug into an active drug at the site of antibody binding.

Examples of imaging agents include radioisotopes generating gamma rays, such as Indium 111 and Technetium 99; radioisotopes generating positrons, such as Copper 64; and passive agents such as Barium which act as contrast agents for X-rays, and Gadolinium in nmr/esr scanning.

In order to link a metallic agent, such as a radioisotope, to a specific binding agent of the invention, it may be necessary to employ a coupling or chelating agent. Many suitable chelating agents have been developed, and reference can be made for example to US 4824986, US 4831175, US 4923985 and US 4622420. Techniques involving the use of chelating agents are described, for example, in US 4454106, US 4722892, Moi et al (1988), McCall et al (1990), Deshpande et al (1990) and Meares et al (1990).

The use of radiolabelled antibodies and fragments in cancer imaging and therapy in humans is described for example in EP 35265. It may be advantageous to use the radiolabelled cancer-specific antibody or fragment in conjunction with a non-specific agent radiolabelled with a different isotope, to provide a contrasting background for so-called subtraction imaging.

The antibody reagents of the invention can be used to identify, e.g. by serum testing or imaging, and/or to treat, PEM-producing cancers. Such cancers can occur as

for example, carcinomas of breast, ovary, uterus and lung, or can manifest themselves as liquids such as pleural effusions.

Modified antibody production

The portions of the VH and VL regions that by convention (Kabat, 1987) are designated as being the CDRs may not be the sole features that need to be transferred from the non-human monoclonal antibody. Sometimes, enhanced antibody performance, in terms of specificity and/or affinity, can be obtained in the reshaped human antibody if certain non-human framework sequences are conserved in the reshaped human antibody. The objective is to conserve the important three-dimensional protein structure associated with the CDRs, which is supported by contacts with framework residues.

The normal starting point from which a reshaped antibody in accordance with the invention can be prepared, is a cell (preferably an immortalised cell line), derived from a non-human host animal (for example, a mouse), which expresses an antibody having specificity against HMFG or PEM. Such a cell line can, for example, be a hybridoma cell line prepared by conventional monoclonal antibody technology. Preferably, the expressed antibody has a high affinity and high specificity for HMFG, because it should be anticipated that some loss of affinity and/or specificity may occur during the transfer of these properties to a human antibody or fragment by the procedures of the invention. By selecting a high specificity antibody as the parent antibody, the likelihood that the final reshaped antibody or fragment will also exhibit effective binding properties is enhanced.

The next stage is the cloning of the cDNA from the cell expressing the selected non-human antibody, and sequencing and identification of the variable region genes including the sequences encoding the CDRs. The experimental procedures involved can now be regarded as routine in the art, although they are still laborious.

If the object is to produce a reshaped complete human antibody, or at least a fragment of such an antibody which will contain both heavy and light variable domains, it will be necessary to sequence the cDNA associated with both of these domains.

Once the relevant cDNA sequence or sequences have been analysed, it is necessary to prepare one or more replicable expression vectors containing a DNA sequence which encodes at least a variable domain of an antibody, which variable domain comprises human framework regions together with one or more CDRs derived from the selected non-human anti-HMFG antibody. The DNA sequence in each vector should include appropriate regulatory sequences necessary to ensure efficient transcription and translation of the gene, particularly a promoter and leader sequence operably linked to the variable domain sequence. In a typical procedure to produce a reshaped antibody or fragment in accordance with the invention, it may be necessary to produce two such expression vectors, one containing a DNA sequence for a reshaped human light chain and the other, a DNA sequence for a reshaped human heavy chain. The expression vectors should be capable of transforming a chosen cell line in which the production of the reshaped antibody or fragment will occur. Such a cell line may be for example, a stable non-producing myeloma cell line, examples (such as NS0 and sp2-0) of which are readily available commercially. An alternative is to use

a bacterial system, such as E.coli, as the expression vehicle for the reshaped antibody or fragment. The final stages of the procedure therefore involve transforming the chosen cell line or organism using the expression vector or vectors, and thereafter culturing the transformed cell line or organism to yield the reshaped human antibody or fragment.

By way of example only, detailed steps by means of which appropriate expression vectors can be prepared are given later in this specification. The manipulation of DNA material in a suitably equipped laboratory is now a well-developed art, and the procedures required are well within the skill of those versed in this art. Many appropriate genomic and cDNA libraries, plasmids, restriction enzymes, and the various reagents and media which are required in order to perform such manipulations, are available commercially from suppliers of laboratory materials. For example, genomic and cDNA libraries can be purchased from Clontech Laboratories Inc. The steps given by way of example below are purely for the guidance of the reader of this specification, and the invention is in no way critically dependant upon the availability of one or more special starting materials. In practice, the skilled person has a wide range of materials from which to choose, and can exploit and adapt the published technology using acquired experience and materials that are most readily available in the scientific environment. For example, many plasmids fall into this category, having been so widely used and circulated within the relevant scientific community that they can now be regarded as common-place materials.

Examples

The procedure used to prepare reshaped anti-HMFG human antibodies is described in detail below, by way of example only, with reference to the accompanying drawings, of which:

Figure 1 shows the cDNA sequence coding for a murine heavy chain variable region having anti-HMFG specificity. The 3 classical CDRs are indicated, together with an amino acid sequence matching the cDNA code.

Figure 2 shows the cDNA sequence coding for a murine light chain variable region having anti-HMFG specificity.

Figure 3a shows a design for a synthesic reshaped human VH gene with HMFG1 specificity (HuVHIconHMFG1 gene cassette) containing 3 fragments.

Figures 3b to 3d show the sequence of the respective fragments in Figure 3a, and also the oligonucleotides used in the assembly of each fragment.

Figures 4a, 4b and 4c together show a route by which an expression vector encoding a reshaped human heavy chain incorporating the CDRs of Figure 1, can be prepared.

Figures 5a and 5b together show a similar transformation route to obtain an expression vector encoding a reshaped human light chain incorporating the CDRs of Figure 2, can be prepared.

Figure 6 shows the plasmid pUC12-IgEnh, which contains an enhancer sequence used in the routes of Figures 4a to 5b.

Figure 7 shows the source of plasmid pBGS18-HulgG1 used in the route of Figure 4c.

Figure 8 shows the source of plasmid pBGS18-HuCk used in the route of Figure 5b.

Figure 9 shows two synthetic oligonucleotide sequences I and II used in cloning the cDNA sequences of Figures 1 and 2.

Figure 10 shows two synthetic oligonucleotide sequences III and IV used to introduce the Kpn I and Sal I restriction sites in M13mp9HuVHLYS respectively, in the route depicted in Figure 4a.

Figure 11 shows three synthetic oligonucleotide sequences VI, VII and VIII used to graft the Vk HMFG1 CDRs onto the human VK REI framework regions in the route depicted in Figure 5a.

Figures 12 and 13 show the cDNA and amino acid sequences of the resulting reshaped human heavy and light chain variable regions respectively.

Figure 14 depicts in diagramatic form the structure of a typical antibody (immunoglobulin) molecule.

Figure 15 shows in graphical form the relative specific anti-HMFG1 binding activity of the resulting reshaped human antibody.

The experimental procedures required to practice the invention do not in themselves represent unusual technology. The cloning and mutagenesis techniques were performed as generally described for example in Verhoeyen

et al (1988); Riechmann et al (1988) and EP-A-239400 (Winter). The "de novo" synthesis of a reshaped human heavy chain variable region gene (see Figures 3a - 3d) was done by conventional techniques, using a set of long overlapping oligonucleotides (see also Jones et al, 1988). Laboratory equipment and reagents for synthesising long oligonucleotides are readily available, and as techniques in this field develop it is becoming practicable to synthesise progressively longer sequences.

Detailed laboratory manuals, covering all basic aspects of recombinant DNA techniques, are available, e.g. "Molecular Cloning" by Sambrook et al (1989).

By means of the invention, the antigen binding regions of a mouse anti-HMFG antibody (HMFG1) were grafted onto human framework regions. The resulting reshaped human antibody (designated HuHMFG1) has binding characteristics similar to those of the original mouse antibody.

Such reshaped antibodies can be used for in vivo diagnosis and treatment of human cancers, eg. ovarian cancers and breast cancers, and are expected at least to reduce the problem of an immune response in the patient often seen upon administration of non-human antibody. A similar benefit has been shown for reshaped CAMPATH-1 antibody in Hale et al (1988).

Methods:

1. Cloning and sequence determination of the mouse variable region genes

Messenger RNA was isolated from a murine hybridoma line which secretes the gamma-1, kappa anti-HMFG antibody "HMFG1" (see Taylor-Papadimitriou et al, 1981 and Arklie et al, 1981). First strand cDNA was synthesised by priming with oligonucleotides I and II (see Figure 9) complementary to the 5' ends of the CH1 and Ck exons respectively. Second strand cDNA was obtained as described by Gübler and Hoffmann (1983).

Kinased EcoRI linkers were ligated to the heavy chain double-stranded cDNA and Pst1 linkers to the light chain double-stranded cDNA (both were first treated with EcoRI or PstI methylase to protect possible internal sites), followed by cloning into EcoRI or PstI-cut pUC9 (Vieira et al, 1982) and transformation of E.coli strain TG2 (Gibson, 1984).

Colonies containing genes coding for murine HMFG1 VH (MoVHHMFG1) and for murine anti-HMFG Vk (MoVkHMFG1) were identified by colony hybridisation with 2 probes consisting respectively of 32P-labelled first strand cDNA of HMFG1 VH and Vk. Positive clones were characterised by plasmid preparation, followed by EcoRI or PstI digestion and 1.5% agarose gel analysis. Full-size inserts (about 450bp) were subcloned in the EcoRI or PstI site of M13mp18 (Norrander et al, 1983). This yielded clones with inserts in both orientations, facilitating nucleotide sequence determination of the entire insert, by the dideoxy chain termination method (Sanger et al, 1977).

The nucleotide sequences, and their translation into amino acid sequences, of the mature variable region genes MoVHHMFG1 and MoVkHMFG1, are shown in Figures 1 and 2. The 450 bp inserts included a signal sequence and 5' untranslated sequences and linkers, not shown in the Figures.

2. Grafting of the mouse HMFG1 CDRs onto human framework regions

The general techniques necessary to achieve this have been described very adequately in Jones et al (1986), Verhoeyen et al (1988), Riechmann et al (1988) and in EP-A-239400 (Winter).

a) Light chain:

The basic construct used for reshaping a human light chain was M13mp9HuVkLYS (Riechmann et al, 1988), which contains framework regions with sequences based on those of the light chain variable regions of the human Bence-Jones protein REI (Epp et al, 1974).

The CDRs in this construct (Figure 5a) were replaced by site-directed mutagenesis with oligonucleotides VI, VII and VIII encoding the HMFG1 kappa chain CDRs flanked by 12 nucleotides at each end encoding the corresponding human framework residues. These oligonucleotides are shown in Figure 11. The mutagenesis was done as described in Riechmann et al (1988). The resulting reshaped human light chain variable region gene (HuVkHMFG1) is shown in Figure 13.

b) Heavy chain:

A reshaped human heavy chain variable region gene was obtained by "de novo" synthesis. In the experiments published by Jones et al, etc, mentioned above, rodent heavy chain CDRs were grafted onto the framework regions of the human NEW heavy chain variable region. It was shown by Verhoeyen et al (1988) and by Riechmann et al (1988) that it is important that the human framework can support the rodent CDRs in a conformation similar to the one occurring in the original rodent antibody, and that certain CDR-framework interactions can be critical. It follows thus that the more dissimilar the rodent and the human framework sequences are, the less the chance will be for the CDR graft to "take".

Comparison of the heavy chain variable region amino acid sequence of the mouse HMFG1 (Figure 1) to that of the human NEW (as used in Verhoeyen et al, 1988), revealed 44% differences between their respective framework regions. A much better homology was found when comparing to human heavy chain variable regions of subgroup I (Kabat et al, 1987); human VHNEW belongs to subgroup II.

We therefore decided to synthesise a human heavy chain variable region gene of subgroup I, containing the HMFG1 heavy chain CDRs. We designed a consensus sequence for human heavy chain subgroup I variable regions, based on sequence information on this subgroup in Kabat et al, 1987. Optimal codon usage was taken from the sequences of mouse constant region genes (the genes are expressed in a mouse myeloma line).

There are only 14% differences between the framework sequences of the HMFG1 VH and the VH of this human VH subgroup I consensus sequence (HuVHIcon). The resulting reshaped gene was designated the name HuVHIconHMFG1, and is depicted in Figure 12. The gene synthesis is described separately in section (c) below. The newly synthesised gene HuVHIconHMFG1 was used to replace HuVHLYS in the construct M13mp9HuVHLYS (Verhoeyen et al, 1988), yielding the vector M13mp9HuVHIconHMFG1 (see Figure 4a).

3. Assembly of reshaped human antibody genes in expression vectors

The next stage involved the use of a murine heavy chain enhancer IgEnh, described in Neuberger et al (1983) where the enhancer is contained in a 1kb Xbal fragment of plasmid pSV-Vμ1. The 700bp Xbal/EcoRI subfragment of this 1kb Xbal fragment is sufficient to confer enhancer activity.

An alternative source of this enhancer is plasmid pSVneoHuVkPLAP (see Fig. 5a), a variation of which has been deposited in an E.coli strain under the Budapest Treaty on 19 April 1990 as NCTC 12390. As deposited, the plasmid also contains a human kappa-chain constant region gene (cloned in the BamH1 site).

The reshaped human genes as prepared in sections 2(a) and 2(b) above, were excised from the M13 vectors as HindIII - BamHI fragments. The heavy chain variable region genes were cloned into a vector based on pSV2gpt (Mulligan et al, 1981) and the light chain variable region genes cloned into a vector based on pSV2neo (Southern et al, 1981) expression vectors, both containing the immunoglobulin heavy chain enhancer IgEnh. In the pSV2gpt

based antibody expression vector (see Fig. 4b - 4c), the Xbal/EcoRI enhancer containing fragment was cloned in the unique EcoRI site of the pSV2gpt vector (after ligating EcoRI linkers to the filled in Xbal end of the fragment).

In the pSVneo based antibody expression vector (see Fig. 5a - 5b), the 1kb Xbal enhancer containing fragment was first cloned into pUC12 (Vieira et al, 1982), yielding the plasmid pUC12-IgEnh, see Figure 6. The enhancer can then be cut out as a 700bp EcoRI/HindIII fragment (either orientation of the enhancer will work). This 700bp EcoRI/HindIII fragment is present in the plasmid pSVneoHuVkPLAP, that we used to clone the HuVkHMFG1-containing fragment described in section 2a, see Fig. 5a and 5b. The HindIII site in the original pSV2neo had been removed. It is possible to use pSV2gpt as an alternative vector for light chain expression, as in practice there is no need for neo selection.

The HuVHIconMFG1 gene was linked to a human gamma 1 constant region (Takahashi et al, 1982), cloned initially as a 8kb HindIII fragment into the HindIII site of pBGS18 (Spratt et al, 1986), and then in the pSV2gpt expression vector as a BamHI fragment (see Figures 4c and 7). It should be noted that in the Takahashi et al (1982) reference there is an error in Figure 1: the last (3') two sites are BamH1 followed by HindIII, and not the converse. This was confirmed by Flanagan et al (1982).

The HuVkHMFG1 gene was linked to a human C kappa constant region (Hieter et al, 1980) also cloned in as a BamHI fragment (see Figures 5b and 8). The source of the human Ck used in Figure 8 is given in Hieter et al (1980). The 12 kb BamH1 fragment from embryonic DNA (cloned in a

gamma Ch28 vector system) was subcloned in the BamH1 site of plasmid pBR322.

4. "de novo" synthesis of the HuVHIconHMFG1 gene

We decided to synthesise a gene encoding a human variable region gene of subgroup I (Kabat et al, 1987), and with the CDRs of VHHMFG1 (Figure 1). In summary, the synthetic gene is designed in such a way that it can substitute the HuVHLYS gene in the existing M13mp9HuVHLYS vector. The M13mp9HuVHLYS was mutagenized to contain a KpnI and SalI site at the appropriate places (see also Figure 4a), to enable cloning of the newly synthesized gene as a KpnI-SalI fragment.

The gene sequence was designed as described above in section 2(b) and is depicted in Figure 12. To facilitate the substitution of this gene for the HuVHLYS gene in M13mp9HuVHLYS (Verhoeyen et al, 1988, see also Figure 4a), 5' and 3' extensions were added to the gene. The 5' extension contains 37 bp of the leader intron and 11 bp of the second half of the leader exon (as in M13mp9HuVHLYS), and has a KpnI site at the very 5' end. The 3' extension contains 38 untranslated nucleotides (as in M13mp9HuVHLYS) and ends in a SalI site.

M13mp9HuVHLYS was modified by site directed mutagenesis with oligonucleotides III and IV to contain a KpnI and SalI site at the appropriate places (see Figure 4a and Figure 10). This vector was named M13mp9HuVHLYS(K,S). This enabled cloning of the HuVHIconHMFG1 gene as a KpnI-SalI fragment in KpnI-SalI cut M13mp9HuVHLYS(K,S) vector.

For practical reasons it was decided to synthesise the gene as three fragments (cassettes), which were then assembled in one complete gene.

Each fragment contains one of the three VHHMFG1 CDRs, and can easily be cloned or removed by using the (existing or newly introduced) unique restriction sites (see Figure 3a). Each fragment was elongated at the 5' and 3' end to create a HindIII and BamHI site respectively, to enable cloning in pEMBL9 (Dente et al, 1983). The coding strand of each fragment was divided in oligonucleotides with an average length of 33 bases. The same was done for the non-coding strand, in such a way that the oligonucleotides overlapped approximately 50% with those of the coding strand.

The sequences of each fragment and of the oligonucleotides used for assembly, are shown in Figures 3b, 3c and 3d.

Before assembling the fragments, the 5' ends of the synthetic oligonucleotides had to be phosphorylated in order to facilitate ligation. Phosphorylation was performed as follows: equimolar amounts (50 pmol) of the oligonucleotides were pooled and kinased in 40 µl reaction buffer with 8 units polynucleotide kinase for 30-45 minutes at 37°C. The reaction was stopped by heating for 5 minutes at 70°C and ethanol precipitation. Annealing was done by dissolving the pellet in 30 µl of a buffer containing: 7 mM TrisCl pH 7.5, 10 mM 2-mercapto-ethanol, 5 mM ATP were added. Subsequently the mixture was placed in a waterbath at 65°C for 5 minutes, followed by cooling to 30°C over a period of 1 hour. MgCl2 was added to a final concentration of 10 mM. T4 DNA-ligase (2.5 units) was added and the mixture was placed at 37°C for 30 min.

(or overnight at 16°C). After this the reaction mixture was heated for 10 minutes at 70°C. After ethanol precipitation the pellet was dissolved in digestion buffer and cut with HindIII and BamHI. The mixture was separated on a 2% agarose gel and the fragment with a length corresponding to the correctly assembled cassette was isolated by electro-elution.

The fragments (1, 2, 3) were ligated in pEMBL9 (cut with HindIII/BamHI), yielding the vectors pUR4107, pUR4108 and pUR4109 respectively. The sequence of the inserts was checked by sequence analysis (in both orientations). Fragment 1 was isolated from pUR4107 by KpnI/XhoI digestion, whilst fragment 2 was isolated from pUR4108 by XhoI/SacI digestion, after which they were ligated in KpnI/SacI cut pUR4109 in a three-fragment ligation. The resulting plasmid was named pUR4110 (see Figure 4a). Sequencing analysis showed that the insert contained the desired HuVHIconHMFG1 gene. This gene was cloned in a pSV2gpt-derived expression vector as depicted in Figures 4b and 4c. The vector pSVgptMoVHLYS-MoIgG1 (Verhoeyen et al, 1988) was used as the source of a pSVgpt-based vector containing the IgEnh enhancer.

5. Expression in myeloma cells

Co-transfection of the expression plasmids pSVgptHuVHIconHMFG1-HuIgG1 and pSVneoHuVkHMFG1-HuCk (Figures 4c and 5b) into NSO myeloma cells was done by electroporation (Potter et al, 1984), after linearisation with PvuI. Transfectomas were selected in mycophenolic acid containing medium to select for cells expressing the gpt gene product, and screened for antibody production and anti-HMFG activity by ELISA assays.

Clones positive for both assays were obtained and subcloned by limiting dilution and pure clones were assayed again for anti-HMFG activity, and the best producing clones were grown in serum-free medium for antibody production.

6. Deposited plasmids

E.coli strains containing plasmids used in the above procedure have been deposited, in accordance with the provisions of the Budapest Treaty, in the National Collection of Type Cultures on 11 July 1990 as follows:

NCTC 12411: K12, TG1 E.coli containing plasmid pSVgptHuVHIconHMFG1-HuIgG1 (identified for the purposes of deposition simply as pSVgpt-HuVHHMFG1-HuIgG1)

NCTC 12412: K12, TG1 E.coli containing plasmid pSVneo-HuVkHMFG1-HuCk

7. Binding ability of the reshaped human antibodies

A useful way of demonstrating binding ability of the reshaped antibody is to show that it has a similar antibody dilution curve when binding to antigen adsorbed on a solid surface. Such curves were generated as follows, using the parent murine anti-HMFG antibody and a reshaped human antibody prepared by the foregoing procedure.

0.5ml of 10% w/v M280 tosyl activated magnetic beads (Dynal, Wirral, UK) were coupled to milk mucin (10^6 units as determined in an immunoassay for HMFG1 in which normal human serum registers 100-200 units per ml). Milk mucin

was prepared from human breast milk according to the method of Burchell et al (1987). The level of mucin was chosen to provide suitable activity for the assays in which the beads were used. The coupling was in 2.5ml of 0.5M borate buffer at pH 9.5 plus 2.5 ml of mucin in phosphate-buffered saline pH 7.2 (PBS) for 22hrs at 37°C with gentle rotation. Blocking of remaining active sites was accomplished by adding 1ml of 10% bovine serum albumen (BSA; Sigma) in PBSA (PBS + 0.02% sodium azide followed by a further 7 hr incubation at 37°C. The excess protein was washed away after using a samarium cobalt magnet to pellet the beads. Further washing was 3x in wash buffer (0.1M potassium phosphate pH 8.0, 0.1% Tween 20, 0.5% BSA) and 4x in rinse buffer (PBS + 0.1% BSA, 0.1% merthiolate). Beads were stored in rinse buffer at 10% w/v (estimated by dry weight analysis).

Antibody binding was measured from a series of doubling dilutions of antibody samples (prepared by weighing in critical cases). 50µl samples were incubated in replicate in microtitre wells with 50µl of 0.05% w/v suspension of beads in 1% BSA/PBSM (PBS + 0.01% merthiolate) at room temperature for 1 hr on a plate shaker. Small cobalt samarium magnets, embedded in a plastic base, were used to sediment the beads to the sides of the wells of the plate to allow liquid removal and washing once with 150µl PBSTM (PBSM + 0.15% Tween 20). This was followed by detection of bound antibody with 50µl of alkaline phosphatase coupled goat anti-human IgG (H+L) (Jackson) used at 1/1000 dilution in 1% BSA in PBSTM for 1 hr at room temperature. The beads were washed 3x in PBSTM. Colour development was with 200µl of nitro phenyl phosphate (Sigma alkaline phosphatase substrate tablets) in 1M diethanolamine buffer at pH 9.8. Optical densities were read in a Dynatech plate reader at 410nm after

transferring fixed volumes of supernatant (usually 150μl) to a flat bottom well microtitre plate. For examination of mouse antibodies the conjugate used was rabbit anti-mouse IgG (Sigma).

Antibody dilution curves for the murine and reshaped HMFG1 antibodies are shown in Figure 15. Maximum binding was determined with a large excess of antibody and negative controls had none. Antibody concentrations, in μg/ml, were determined by UV absorption measurements at 280nm. For both antibodies a dilution of 1 has been set equivalent to 1μg/ml. The two curves are similar, indicating a significant and useful level of binding effectiveness for the reshaped antibody of the invention.

References:

Arklie et al (1981) - Int. J. Cancer, 28, p.23-29

Burchell et al (1987) - Cancer Res., 47, p.5476

Dente et al (1983) - Nucleic Acids Res. II, p.1645-1655

Epp et al (1974) - Eur. J. Biochem. 45, p.513-524

Flanagan et al (1982) - Nature, 300, p.709-713

Gendler et al (1988) - J. Biol. Chem, 236, p.12820-12823

Gibson T (1984) - PhD thesis, LMB-MRC Cambridge

Gubler et al (1983) - Gene, 25, p.263-269

Hale et al (1988) - Lancet, 2, p.1394

Hieter et al (1980) - Cell, 22, p.197-207

Jones et al (1986) - Nature, 321, p.522-525

Kabat et al (1987) - in Sequences of Proteins of Immunological Interest, p.ix -US Dept of Health and Human Services

Mulligan et al (1981) - Proc. natn. Acad. Sci. U.S.A., 78 p.2072-2076

Neuberger et al (1983) - EMBO Journal, 2, p.1373-1378

Norrander et al (1983) - Gene, 26, p.101-106

Potter et al (1984) - PNAS, 81, p.7161-7163

Riechmann et al (1988) - Nature, 332, p.323-327
Sambrook et al (1989) - Molecular Cloning, 2nd Edition, Cold Spring Harbour Laboratory Press, New York
Sanger et al (1977) - PNAS USA, 74, p.5463-5467
Saul et al (1978) - J. biol. Chem. 253, p.585-597
Southern et al (1981) - J. molec. appl. Genetics, 1 p.327-345
Spratt et al (1936) - Gene, 41, p.337-342
Takahashi et al (1982) - Cell, 29, p.671-679
Taylor-Papadimitrion et al (1981) - Int. J. Cancer, 28, p.17-21
Verhoeyen et al (1988) - Science, 239, p.1534-1536
Vieira et al (1982) - Gene, 19, p.259-268
Winter (1987) - EP-A-239400
Xing et al (1990) - EP-A2-369816

CLAIMS

1. A synthetic specific binding agent having specificity for human polymorphic epithelial mucin (PEM), conferred by the presence of one or more of the amino acid sequences:

i) Ala Tyr Trp Ile Glu

ii) Glu Ile Leu Pro Gly Ser Asn Asn Ser Arg Tyr Asn Glu Lys Phe Lys Gly

iii) Ser Tyr Asp Phe Ala Trp Phe Ala Tyr

iv) Lys Ser Ser Gln Ser Leu Leu Tyr Ser Ser Asn Gln Lys Ile Tyr Leu Ala

v) Trp Ala Ser Thr Arg Glu Ser

vi) Gln Gln Tyr Tyr Arg Tyr Pro Arg Thr

2 A reshaped human antibody, or a reshaped human antibody fragment, having specificity for human polymorphic epithelial mucin (PEM) conferred by the presence of one or more of the amino acid sequences:

i) Ala Tyr Trp Ile Glu

ii) Glu Ile Leu Pro Gly Ser Asn Asn Ser Arg Tyr Asn Glu Lys Phe Lys Gly

iii) Ser Tyr Asp Phe Ala Trp Phe Ala Tyr

iv) Lys Ser Ser Gln Ser Leu Leu Tyr Ser Ser Asn Gln Lys Ile Tyr Leu Ala

v) Trp Ala Ser Thr Arg Glu Ser

vi) Gln Gln Tyr Tyr Arg Tyr Pro Arg Thr

3. A reshaped human antibody or reshaped human antibody fragment according to claim 2, having at least one heavy-chain variable region incorporating the following CDRs:

CDR1: Ala Tyr Trp Ile Glu

CDR2: Glu Ile Leu Pro Gly Ser Asn Asn Ser Arg Tyr Asn Glu Lys Phe Lys Gly

CDR3: Ser Tyr Asp Phe Ala Trp Phe Ala Tyr

4. A reshaped human antibody or reshaped human antibody fragment according to claim 2, having at least one light-chain variable region incorporating the following CDRs:

CDR1: Lys Ser Ser Gln Ser Leu Leu Tyr Ser Ser Asn Gln Lys Ile Tyr Leu Ala

CDR2: Trp Ala Ser Thr Arg Glu Ser

CDR3: Gln Gln Tyr Tyr Arg Tyr Pro Arg Thr

5. A reshaped human antibody or reshaped human antibody fragment according to claim 2 and having at least one heavy-chain variable region according to claim 3 and at least one light-chain variable region according to claim 4.

6. A reshaped human antibody or reshaped human antibody fragment according to claim 2, incorporating at least one heavy-chain variable region comprising the entire amino acid sequence depicted in Figure 12 of the accompanying drawings.

7. A reshaped human antibody or reshaped human antibody fragment according to claim 2, incorporating at least one light-chain variable region comprising the entire amino acid sequence depicted in Figure 13 of the accompanying drawings.

8. A synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, according to any one of the preceding claims, wherein the PEM is human milk fat globule (HMFG).

9. A synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, having specificity equivalent to that of the gamma-1, kappa anti-HMFG monoclonal antibody "HMFG1".

10. A stable host cell line producing a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment according to any one of claims 1 to 9, resulting from incorporation in the cell line of a foreign gene encoding the synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment.

11. A stable host cell line according to claim 10, wherein the foreign gene includes one or more of the nucleotide sequences:

i) GCC TAC TGG ATA GAG

ii) GAG ATT TTA CCT GGA AGT AAT AAT TCT AGA TAC AAT GAG AAG TTC AAG GGC

iii) TCC TAC GAC TTT GCC TGG TTT GCT TAC

iv) AAG TCC AGT CAG AGC CTT TTA TAT AGT AGC AAT CAA AAG ATC TAC TTG GCC

v) TGG GCA TCC ACT AGG GAA TCT

vi) CAG CAA TAT TAT AGA TAT CCT CGG ACG

12. A stable host cell line according to claim 10, wherein the foreign gene includes the entire nucleotide sequence depicted in Figure 12 of the accompanying drawings.

13. A stable host cell line according to claim 10, wherein the foreign gene includes the entire nucleotide sequence depicted in Figure 13 of the accompanying drawings.

14. A stable host cell line according to claim 10, wherein the foreign gene encodes:

a) at least one of the amino acid sequences:

i) Ala Tyr Trp Ile Glu

ii) Glu Ile Leu Pro Gly Ser Asn Asn Ser Arg Tyr Asn Glu Lys Phe Lys Gly

iii) Ser Tyr Asp Phe Ala Trp Phe Ala Tyr

iv) Lys Ser Ser Gln Ser Leu Leu Tyr Ser Ser Asn Gln Lys

Ile Tyr Leu Ala

v) Trp Ala Ser Thr Arg Glu Ser

vi) Gln Gln Tyr Tyr Arg Tyr Pro Arg Thr

and b) a protein framework that enables the encoded amino acid sequence when expressed to function as a CDR having specificity for PEM.

15. A stable host cell line according to claim 10, wherein the foreign gene encodes the entire amino acid sequence depicted in Figure 12 of the accompanying drawings.

16. A stable host cell line according to claim 10, wherein the foreign gene encodes the entire amino acid sequence depicted in Figure 13 of the accompanying drawings.

17. Plasmid pSVgpt-HuVHHMFG1-HuIgG1.

18. Plasmid pSVneo-HuVkHMFG1-HuCk.

19. Use of plasmid according to claim 17 or claim 18 in the production of a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment.

20. E.coli NCTC 12411.

21. E.coli NCTC 12412.

22. A DNA sequence encoding a reshaped human antibody heavy-chain variable region having specificity for HMFG, as contained in E.coli NCTC 12411.

23. A DNA sequence encoding a reshaped human antibody light-chain variable region having specificity for HMFG, as contained in E.coli NCTC 12412.

24. A reshaped human antibody heavy-chain variable region having specificity for HMFG, producible by means of the expression vector contained in E.coli NCTC 12411.

25. A reshaped human antibody light-chain variable region having specificity for HMFG, producible by means of the expression vector contained in E.coli NCTC 12412.

26. A reshaped human antibody or reshaped human antibody fragment, comprising at least one variable region according to claim 24 or claim 25.

27. A synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, according to any one of claims 1 to 9 or claim 26, linked to or incorporating an agent capable of retarding or terminating the growth of cancerous cells, or linked to an agent capable of being detected while inside the human body.

28. An injectable composition comprising a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, according to claim 27, in a pharmaceutically acceptable carrier.

29. Use of a synthetic specific binding agent, reshaped human antibody or reshaped human antibody fragment, according to any one of claims 1 to 9 or claim 26, for the

manufacture of a medicament for therapeutic application in the relief of cancer in humans, or for the manufacture of a diagnostic composition for in-vivo diagnostic application in humans.

30. Use of a synthetic binding agent, reshaped human antibody or reshaped human antibody fragment, according to claim 27, in a method of human cancer therapy or imaging.

Fig. 1.

MoVHHMFG1

```
                    5                  10                  15                  20
CAG GTT CAG CTG CAG CAG TCT GGA GCT GAG CTG ATG AAG CCT GGG GCC TCA GTG AAG ATA      60
Gln Val Gln Leu Gln Gln Ser Gly Ala Glu Leu Met Lys Pro Gly Ala Ser Val Lys Ile

                    25                  30        CDR1      35                  40
TCC TGC AAG GCT ACT GGC TAC ACA TTC AGT GCC TAC TGG ATA GAG TGG GTA AAG CAG AGG     120
Ser Cys Lys Ala Thr Gly Tyr Thr Phe Ser Ala Tyr Trp Ile Glu Trp Val Lys Gln Arg

                    45                  50    52  A           55        CDR2
CCT GGA CAT GGC CTT GAG TGG ATT GGA GAG ATT TTA CCT GGA AGT AAT AAT TCT AGA TAC     180
Pro Gly His Gly Leu Glu Trp Ile Gly Glu Ile Leu Pro Gly Ser Asn Asn Ser Arg Tyr

60                      65                  70                  75
AAT GAG AAG TTC AAG GGC AAG GCC ACA TTC ACT GCT GAT ACA TCC TCC AAC ACA GCC TAC     240
Asn Glu Lys Phe Lys Gly Lys Ala Thr Phe Thr Ala Asp Thr Ser Ser Asn Thr Ala Tyr

80      82  A   B   C           85                  90                  95
ATG CAA CTC AGC AGC CTG ACA TCT GAG GAC TCT GCC GTC TAT TAC TGT TCA AGG TCC TAC     300
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ser Arg Ser Tyr

 CDR3       100  A                  105                 110
GAC TTT GCC TGG TTT GCT TAC TGG GGC CAA GGG ACT CCG GTC ACT GTC TCT GCA            354
Asp Phe Ala Trp Phe Ala Tyr Trp Gly Gln Gly Thr Pro Val Thr Val Ser Ala
```

Fig.2.

MoVkHMFG1

```
                    5                  10                  15                  20
GAC ATT GTG ATG TCA CAG TCT CCA TCC TCC CTA GCT GTG TCA GTT GGA GAG AAG GTT ACT     60
Asp Ile Val Met Ser Gln Ser Pro Ser Ser Leu Ala Val Ser Val Gly Glu Lys Val Thr

                   25      27  A   B   C   D   E   F          30        CDR1
ATG AGC TGC AAG TCC AGT CAG AGC CTT TTA TAT AGT AGC AAT CAA AAG ATC TAC TTG GCC    120
Met Ser Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser Ser Asn Gln Lys Ile Tyr Leu Ala

 35                  40                  45                  50        CDR2
TGG TAC CAG CAG AAA CCA GGG CAG TCT CCT AAA CTG CTG ATT TAC TGG GCA TCC ACT AGG    180
Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr Arg

 55                  60                  65                  70
GAA TCT GGG GTC CCT GAT CGC TTC ACA GGC GGT GGA TCT GGG ACA GAT TTC ACT CTC ACC    240
Glu Ser Gly Val Pro Asp Arg Phe Thr Gly Gly Gly Ser Gly Thr Asp Phe Thr Leu Thr

 75                  80                  85                  90        CDR3
ATC AGC AGT GTG AAG GCT GAA GAC CTG GCA GTT TAT TAC TGT CAG CAA TAT TAT AGA TAT    300
Ile Ser Ser Val Lys Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Arg Tyr

 95                  100                 105
CCT CGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA CGG                           342
Pro Arg Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg
```

Fig.3a

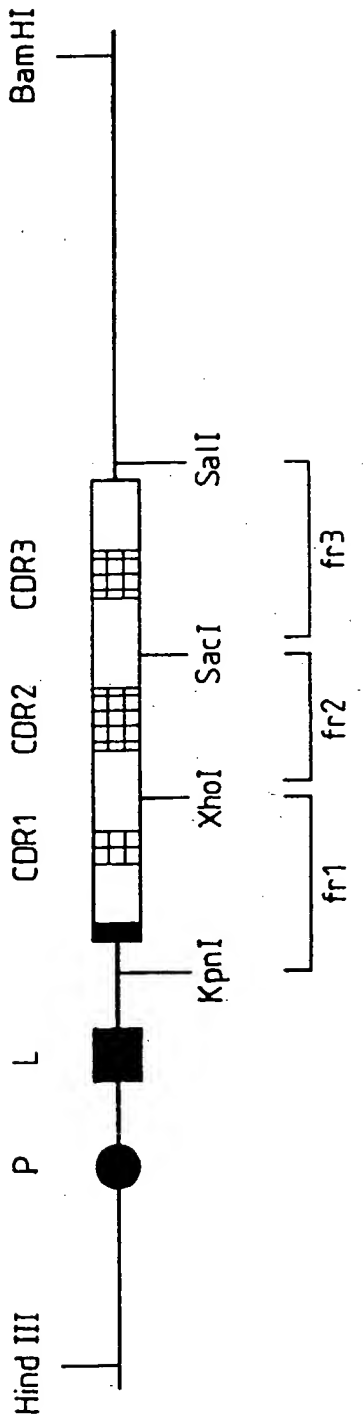

BamHI
SalI
SacI
XhoI
KpnI
CDR3
CDR2
CDR1
L
P
Hind III
fr3
fr2
fr1

Fig. 3b

FRAGMENT 1

```
        10          20          30          40          50          60
acagtagcag gcttgaggaa agcttctata tatgggtacc aatgacatcc actttgcctt
tgtcatcgtc cgaactcctt tcgaagatat atacccatgg ttactgtagg tgaaacggaa

        70          80          90         100         110         120
tctctccaca gGTGTCCACT CCCAGGTGCA GCTGGTGCAG TCTGGGGCAG AGGTGAAAAA
agagaggtgt cCACAGGTGA GGGTCCACGT CGACCACGTC AGACCCCGTC TCCACTTTTT

       130         140         150         160         170         180
GCCTGGGGCC TCAGTGAAGG TGTCCTGCAA GGCTTCTGGC TACACCTTCA GTGCCTACTG
CGGACCCCGG AGTCACTTCC ACAGGACGTT CCGAAGACCG ATGTGGAAGT CACGGATGAC

       190         200         210         220         230         240
GATAGAGTGG GTGCGCCAGG CTCCAGGAAA GGGCCTCGAG TGGGTCGGAT CCAGGGAGAT
CTATCTCACC CACGCGGTCC GAGGTCCTTT CCCGGAGCTC ACCCAGCCTA GGTCCCTCTA
```

OLIGONUCLEOTIDES

CODE	LENGTH	5' ←--- SEQUENCE ---→ 3'
VHHM1A	(32)	agc ttc tat ata tgg gta cca atg aca tcc ac
VHHM1B	(33)	ttt gcc ttt ctc tcc aca gGT GTC CAC TCC CAG
VHHM1C	(36)	GTG CAG CTG GTG CAG TCT GGG GCA GAG GTG AAA AAG
VHHM1D	(33)	CCT GGG GCC TCA GTG AAG GTG TCC TGC AAG GCT
VHHM1E	(36)	TCT GGC TAC ACC TTC AGT GCC TAC TGG ATA GAG TGG
VHHM1F	(37)	GTG CGC CAG GCT CCA GGA AAG GGC CTC GAG TGG GTC G
VHHM1G	(40)	gag aaa ggc aaa gtg gat gtc att ggt acc cat ata tag a
VHHM1H	(36)	CTG CAC CAG CTG CAC CTG GGA GTG GAC ACc tgt gga
VHHM1I	(33)	TGA GGC CCC AGG CTT TTT CAC CTC TGC CCC AGA
VHHM1J	(33)	GGT GTA GCC AGA AGC CTT GCA GGA CAC CTT CAC
VHHM1K	(36)	AGC CTG GCG CAC CCA CTC TAT CCA GTA GGC ACT GAA
VHHM1L	(29)	GAT CCG ACC CAC TCG AGG CCC TTT CCT GG

POSITIVE STRING:

VHHM1A : (21-52)
VHHM1B : (53-85)
VHHM1C : (86-121)
VHHM1D : (122-154)
VHHM1E : (155-190)
VHHM1F : (191-227)

NEGATIVE STRING:

VHHM1G : (25-64)
VHHM1H : (65-100)
VHHM1I : (101-133)
VHHM1J : (134-166)
VHHM1K : (167-202)
VHHM1L : (203-231)

Fig.3c.

FRAGMENT 2

```
        10          20          30          40          50          60
GACAGCCGTA GAGTGGGTGC AAGCTTCTCC AGGACTCGAG TGGGTCGGAG AGATTTTACC
CTGTCGGCAT CTCACCCACG TTCGAAGAGG TCCTGAGCTC ACCCAGCCTC TCTAAAATGG

        70          80          90          100         110         120
TGGAAGTAAT AATTCTAGAT ACAATGAGAA GTTCAAGGGC CGAGTGACAG TCACTAGAGA
ACCTTCATTA TTAAGATCTA TGTTACTCTT CAAGTTCCCG GCTCACTGTC AGTGATCTCT

        130         140         150         160         170         180
CACATCCACA AACACAGCCT ACATGGAGCT CAGCAGCCTG AGGATCCAGC AGCCTGAGGT
GTGTAGGTGT TTGTGTCGGA TGTACCTCGA GTCGTCGGAC TCCTAGGTCG TCGGACTCCA
```

OLIGONUCLEOTIDES

CODE	LENGTH	5' ←--- SEQUENCE ---→ 3'
VHHM2A	(25)	AGC TTC TCC AGG ACT CGA GTG GGT C
VHHM2B	(27)	GGA GAG ATT TTA CCT GGA AGT AAT AAT
VHHM2C	(39)	TCT AGA TAC AAT GAG AAG TTC AAG GGC CGA GTG ACA GTC
VHHM2D	(30)	ACT AGA GAC ACA TCC ACA AAC ACA GCC TAC
VHHM2E	(20)	ATG GAG CTC AGC AGC CTG AG
VHHM2F	(36)	AGG TAA AAT CTC TCC GAC CCA CTC GAG TCC TGG AGA
VHHM2G	(39)	GCC CTT GAA CTT CTC ATT GTA TCT AGA ATT ATT ACT TCC
VHHM2H	(24)	TGT GTC TCT AGT GAC TGT CAC TCG
VHHM2I	(42)	GAT CCT CAG GCT GCT GAG CTC CAT GTA GGC TGT GTT TGT GGA

POSITIVE STRING:

```
VHHM2A    : (22-46)
VHHM2B    : (47-73)
VHHM2C    : (74-112)
VHHM2D    : (113-142)
VHHM2E    : (143-162)
```

NEGATIVE STRING:

```
VHHM2F    : (26-61)
VHHM2G    : (62-100)
VHHM2H    : (101-124)
VHHM2I    : (125-166)
```

Fig. 3d

FRAGMENT 3

```
        10          20          30          40          50          60
CACATCCACA AGCTTAAACA CAGCCGAGCT CAGCAGCCTG AGGTCTGAGG ACACAGCCGT
GTGTAGGTGT TCGAATTTGT GTCGGCTCGA GTCGTCGGAC TCCAGACTCC TGTGTCGGCA

        70          80          90         100         110         120
CTATTACTGT GCAAGATCCT ACGACTTTGC CTGGTTTGCT TACTGGGGCC AAGGGACTCT
GATAATGACA CGTTCTAGGA TGCTGAAACG GACCAAACGA ATGACCCCGG TTCCCTGAGA

      130         140         150         160         170         180
GGTCACAGTC TCCTCAggtg agtccttaca acctctctct tctattcagt cgacatagat
CCAGTGTCAG AGGAGTccac tcaggaatgt tggagagaga agataagtca gctgtatcta

      190
acgtggatcc
tgcacctagg
```

OLIGONUCLEOTIDES

CODE	LENGTH	5' ←--- SEQUENCE ---→ 3'
VHHM3A	(39)	AGC TTA AAC ACA GCC GAG CTC AGC AGC CTG AGG TCT GAG
VHHM3B	(27)	GAC ACA GCC GTC TAT TAC TGT GCA AGA
VHHM3C	(39)	TCC TAC GAC TTT GCC TGG TTT GCT TAC TGG GGC CAA GGG
VHHM3D	(39)	ACT CTG GTC ACA GTC TCC TCA ggt gag tcc tta caa cct
VHHM3E	(31)	ctc tct tct att cag tcg aca tag ata cgt g
VHHM3F	(17)	GAG CTC GGC TGT GTT TA
VHHM3G	(33)	ATA GAC GGC TGT GTC CTC AGA CCT CAG GCT GCT
VHHM3H	(39)	GTA AGC AAA CCA GGC AAA GTC GTA GGA TCT TGC ACA GTA
VHHM3I	(36)	acc TGA GGA GAC TGT GAC CAG AGT CCC TTG GCC CCA
VHHM3J	(29)	tga ata gaa gag aga ggt tgt aag gac tc
VHHM3K	(21)	gat cca cgt atc tat gtc gac

POSITIVE STRING:

VHHM3A : (11-49)
VHHM3B : (50-76)
VHHM3C : (77-115)
VHHM3D : (116-154)
VHHM3E : (155-185)

NEGATIVE STRING:

VHHM3F : (15-31)
VHHM3G : (32-64)
VHHM3H : (65-103)
VHHM3I : (104-139)
VHHM3J : (140-168)
VHHM3K : (169-189)

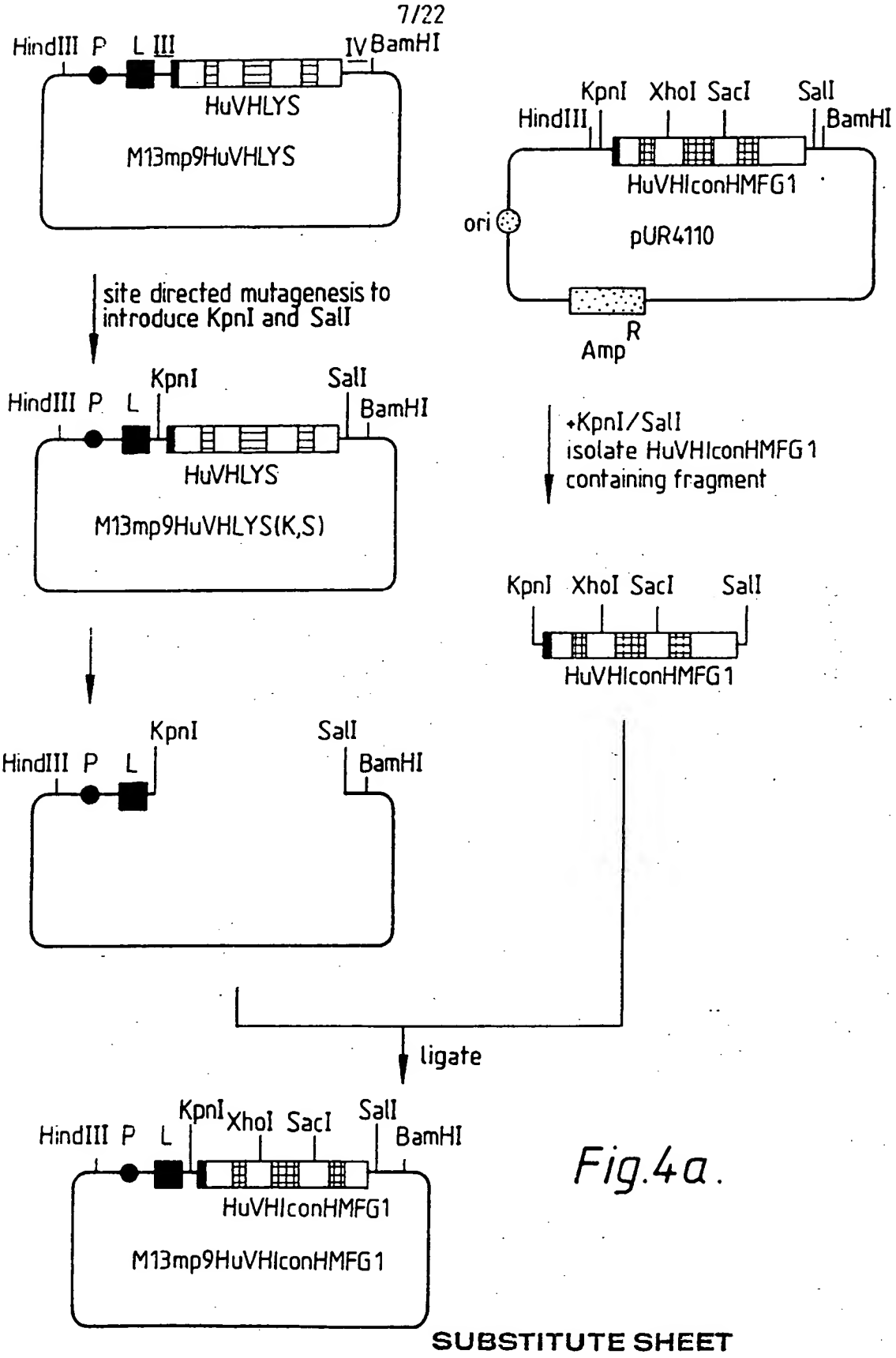

HindIII P L III IV BamHI
HuVHLYS
M13mp9HuVHLYS
site directed mutagenesis to introduce KpnI and SalI
HindIII P L KpnI SalI BamHI
HuVHLYS
M13mp9HuVHLYS(K,S)
HindIII P L KpnI SalI BamHI
HindIII P L KpnI XhoI SacI SalI BamHI
HuVHIconHMFG1
M13mp9HuVHIconHMFG1
KpnI XhoI SacI SalI
HindIII KpnI XhoI SacI SalI BamHI
HuVHIconHMFG1
ori
pUR4110
R
Amp
+KpnI/SalI
isolate HuVHIconHMFG1
containing fragment
ligate

Fig.4a.

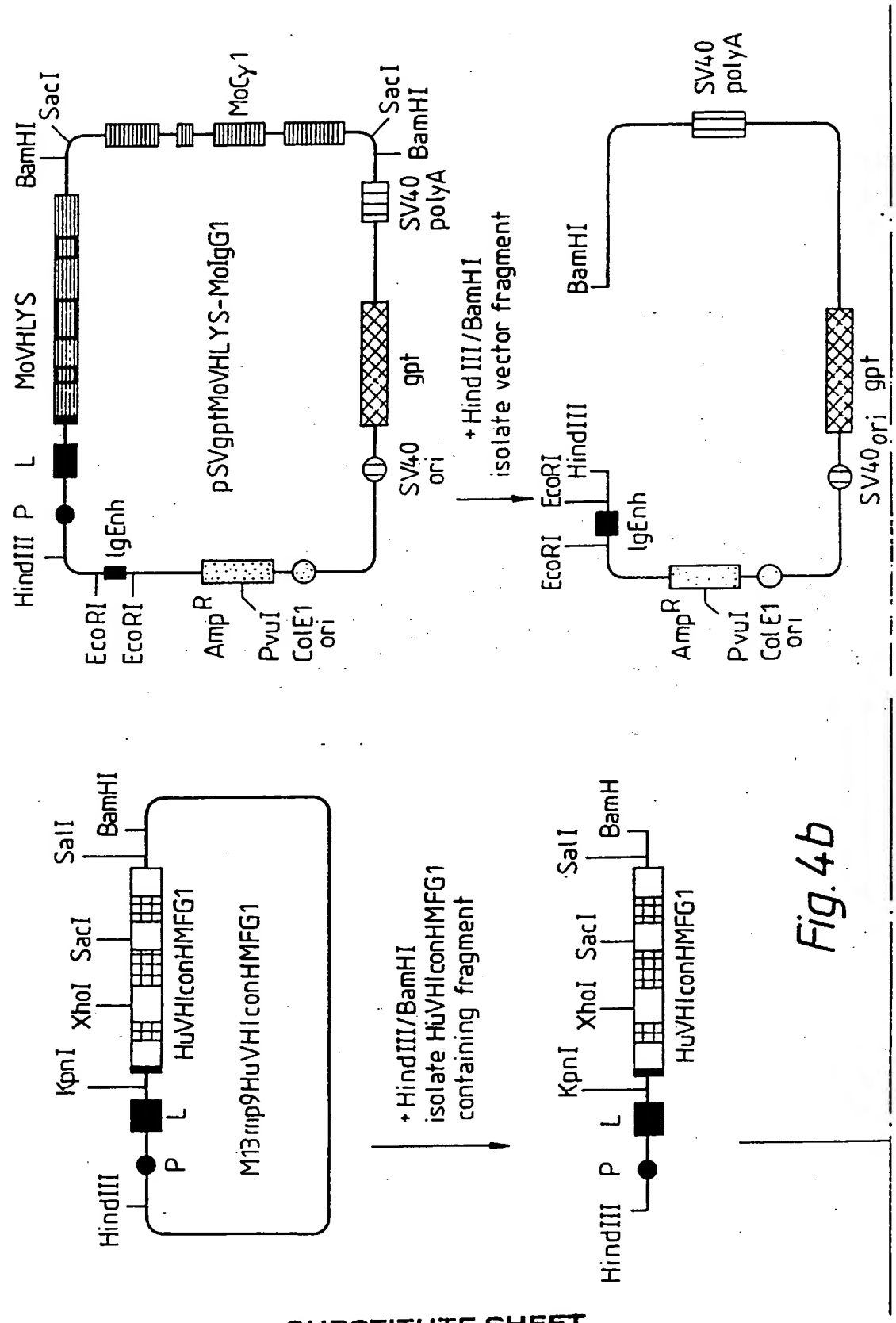
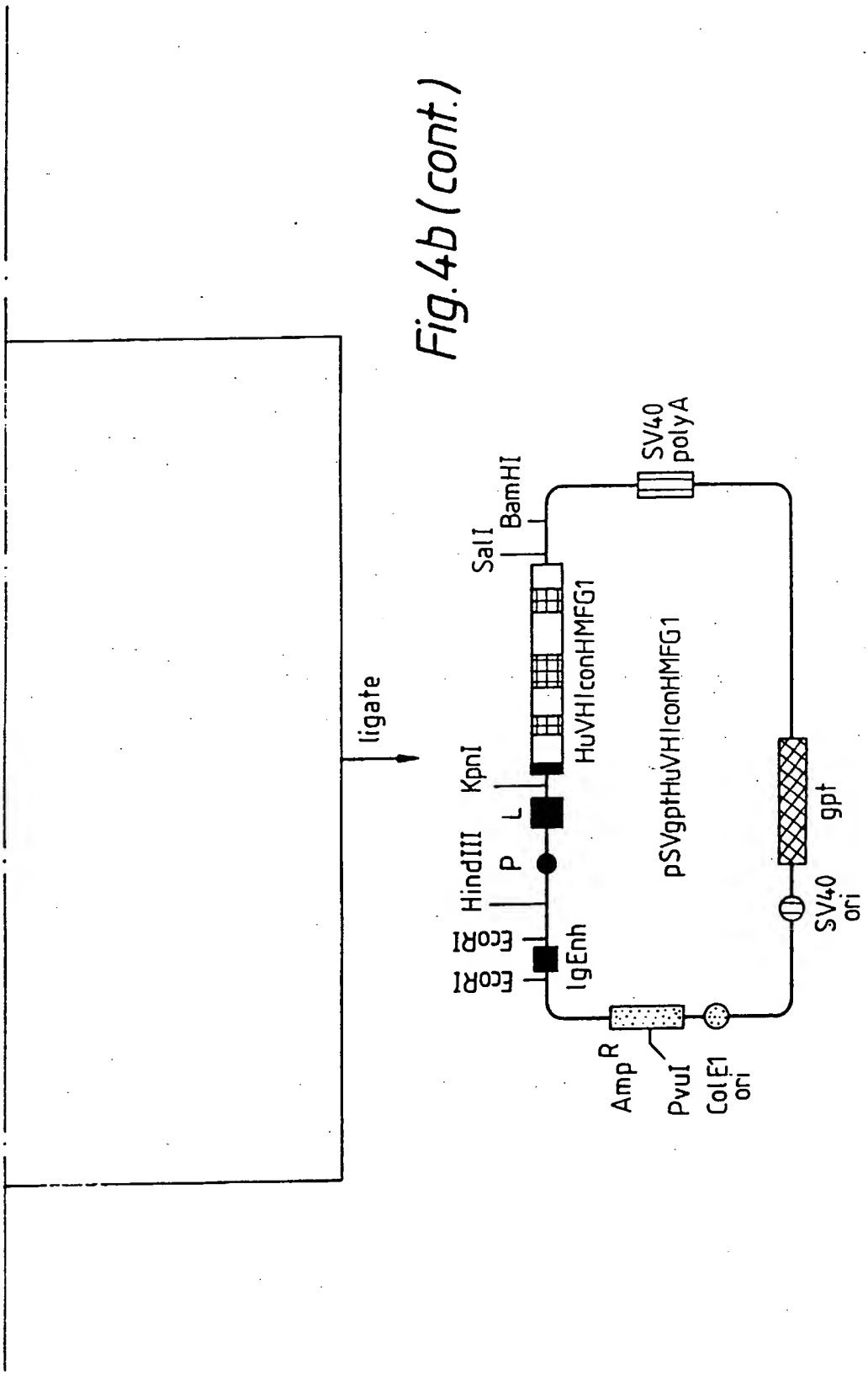

HindIII
P
L
MoVHLYS
BamHI
SacI
EcoRI
EcoRI
IgEnh
pSVgptMoVHLYS-MoIgG1
MoCγ1
SacI
BamHI
SV40 polyA
SV40 ori
gpt
Amp R
PvuI
ColE1 ori
+HindIII/BamHI isolate vector fragment
EcoRI
HindIII
EcoRI
IgEnh
BamHI
SV40 polyA
SV40 ori
gpt
Amp R
PvuI
ColE1 ori
HindIII
P
L
M13mp9HuVHIconHMFG1
KpnI
XhoI
SacI
SalI
BamHI
HuVHIconHMFG1
+HindIII/BamHI isolate HuVHIconHMFG1 containing fragment
HindIII
P
L
KpnI
XhoI
SacI
SalI
BamH
HuVHIconHMFG1

Fig. 4b

Fig. 4b (cont.)


ligate
SV40 polyA
BamHI
SalI
HuVHIconHMFG1
KpnI
L
P
HindIII
EcoRI
EcoRI
IgEnh
pSVgptHuVHIconHMFG1
gpt
SV40 ori
Amp R
PvuI
ColE1 ori

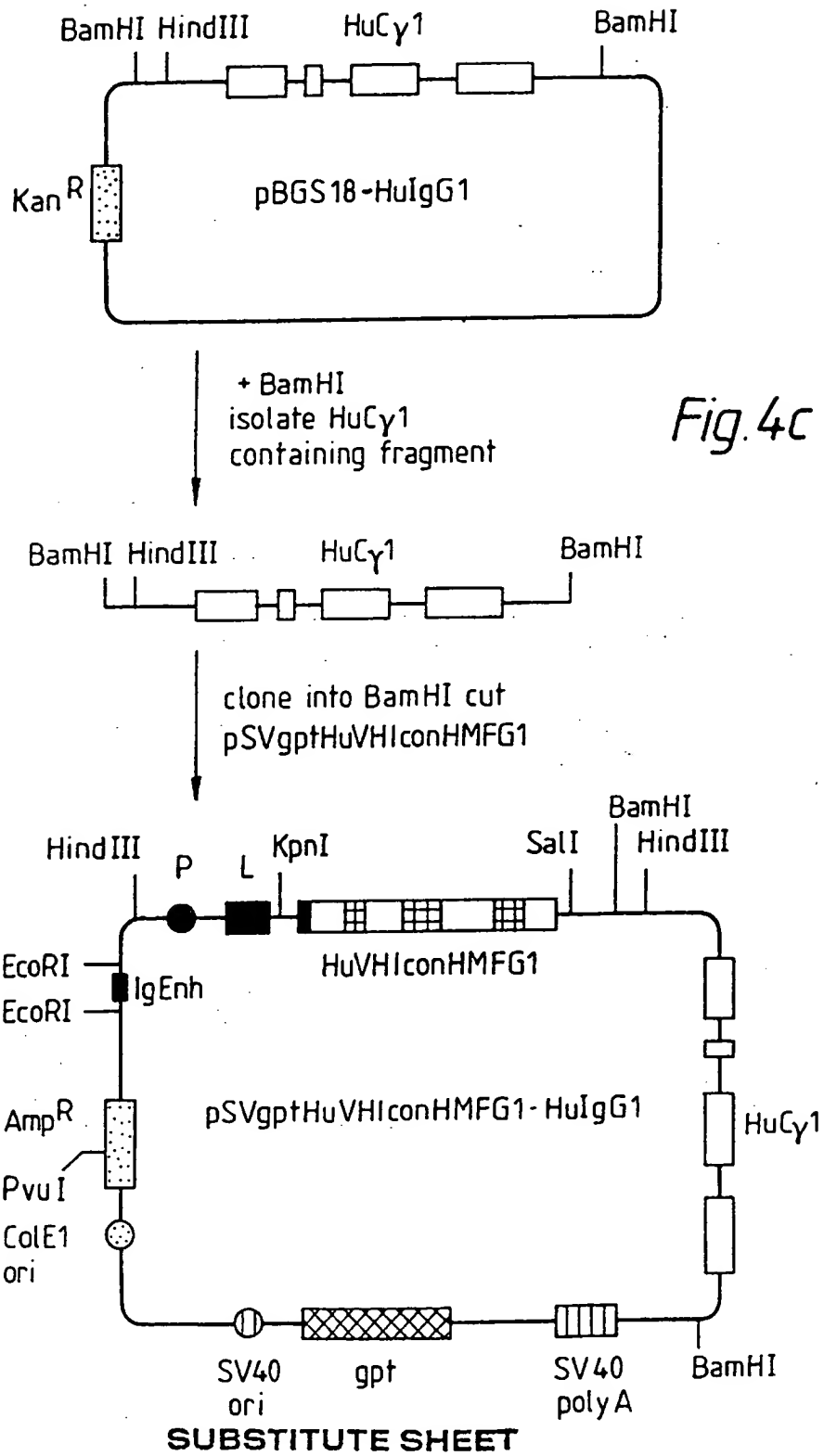

BamHI HindIII
HuCγ1
BamHI
Kan R
pBGS18-HuIgG1
+ BamHI
isolate HuCγ1
containing fragment
BamHI HindIII
HuCγ1
BamHI
clone into BamHI cut
pSVgptHuVHIconHMFG1
BamHI
HindIII
P
L
KpnI
SalI
BamHI
HindIII
EcoRI
EcoRI
IgEnh
HuVHIconHMFG1
Amp R
Pvu I
ColE1
ori
pSVgptHuVHIconHMFG1-HuIgG1
HuCγ1
SV40
ori
gpt
SV40
poly A
BamHI

Fig. 4c

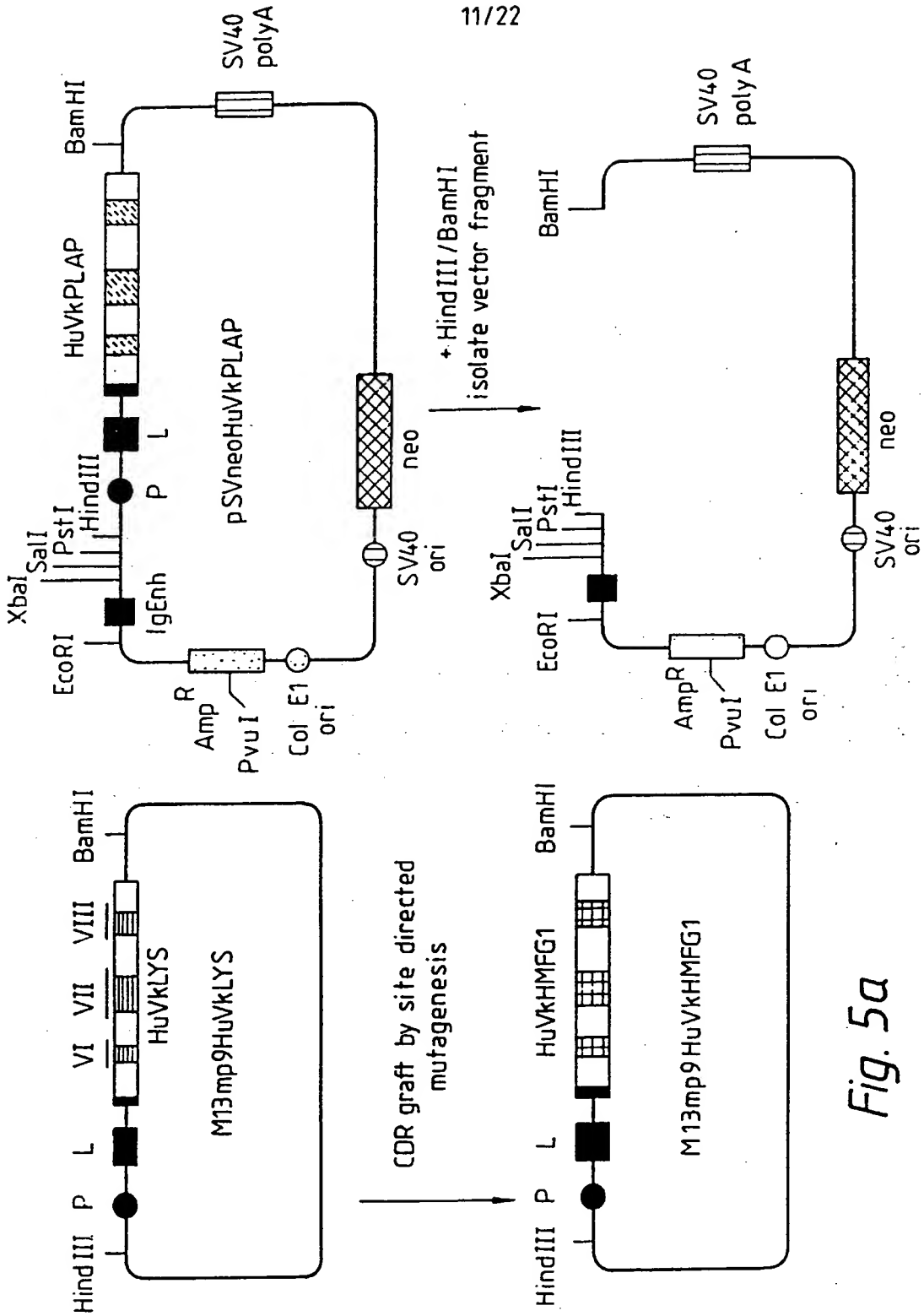
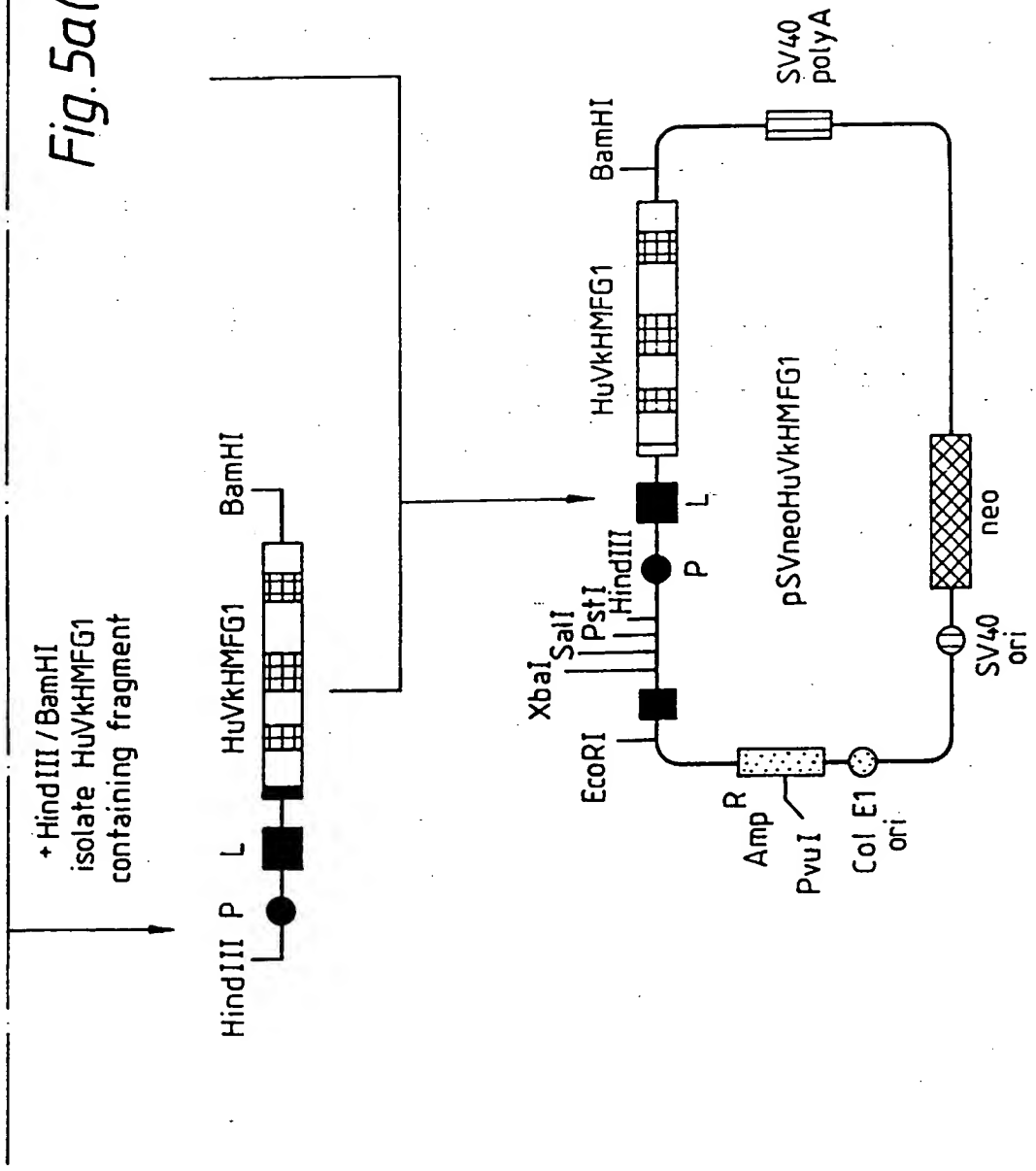
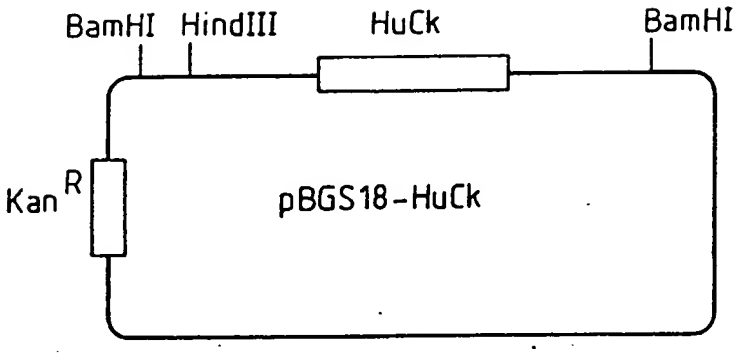

SV40 polyA
BamHI
HuVkPLAP
pSVneoHuVkPLAP
HindIII
PstI
SalI
XbaI
EcoRI
IgEnh
P
L
neo
SV40 ori
Amp R
PvuI
Col E1 ori
+ HindIII/BamHI isolate vector fragment
M13mp9HuVkLYS
HuVkLYS
VI
VII
VIII
COR graft by site directed mutagenesis
M13mp9HuVkHMFG1
HuVkHMFG1

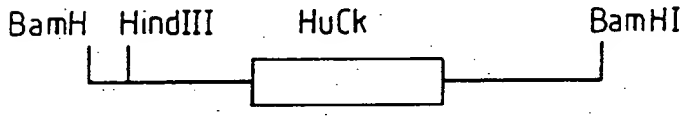
Fig. 5a

Fig. 5a (cont.)


+ HindIII / BamHI isolate HuVkHMFG1 containing fragment
HindIII
P
L
HuVkHMFG1
BamHI
XbaI
SalI
PstI
HindIII
P
L
HuVkHMFG1
BamHI
SV40 polyA
pSVneoHuVkHMFG1
neo
SV40 ori
EcoRI
R
Amp
PvuI
Col E1 ori



+ BamHI
isolate HuCk containing
fragment



clone into BamHI cut
pSVneoHuVkHMFG1

Fig. 5b.

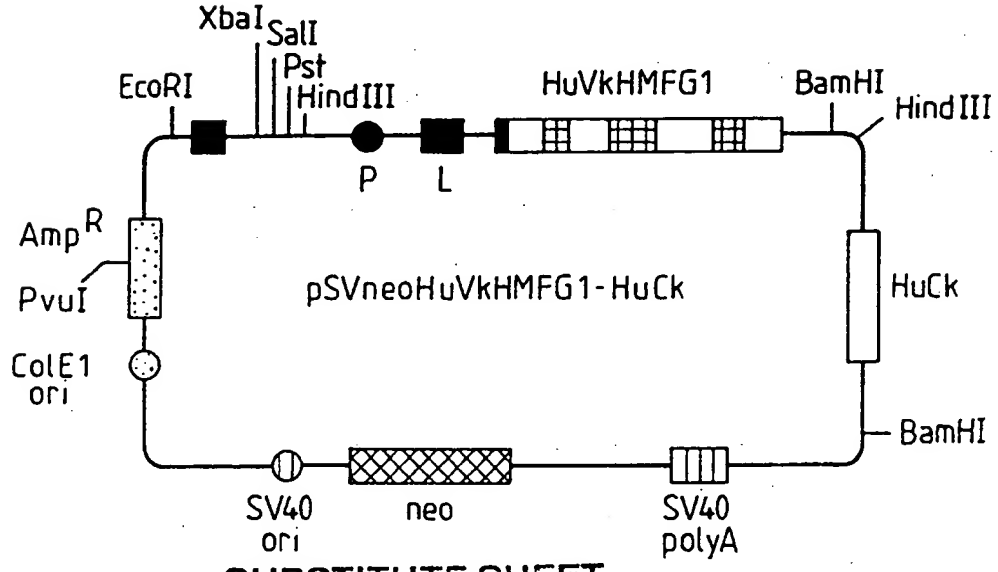


Fig.6.

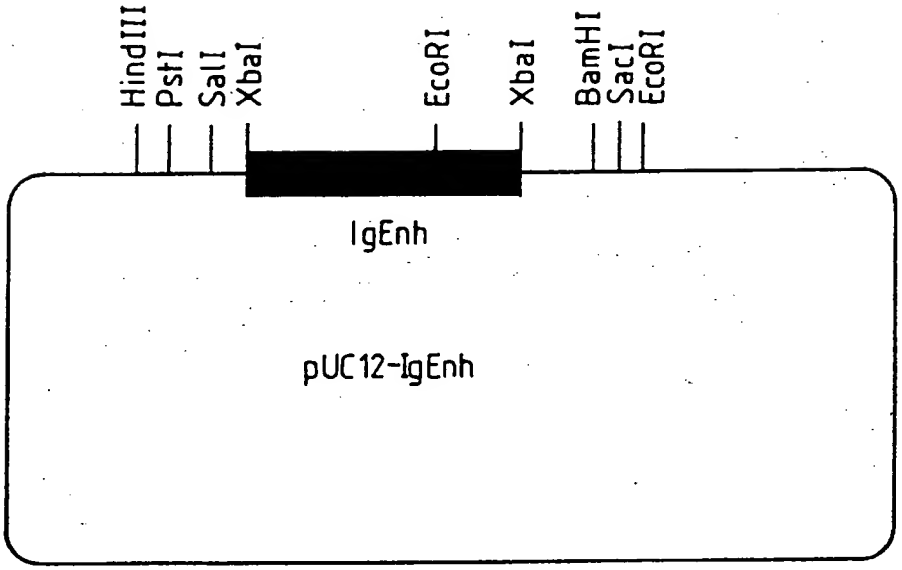

HindIII
PstI
SalI
XbaI
EcoRI
XbaI
BamHI
SacI
EcoRI
IgEnh
pUC12-IgEnh

Fig. 7.

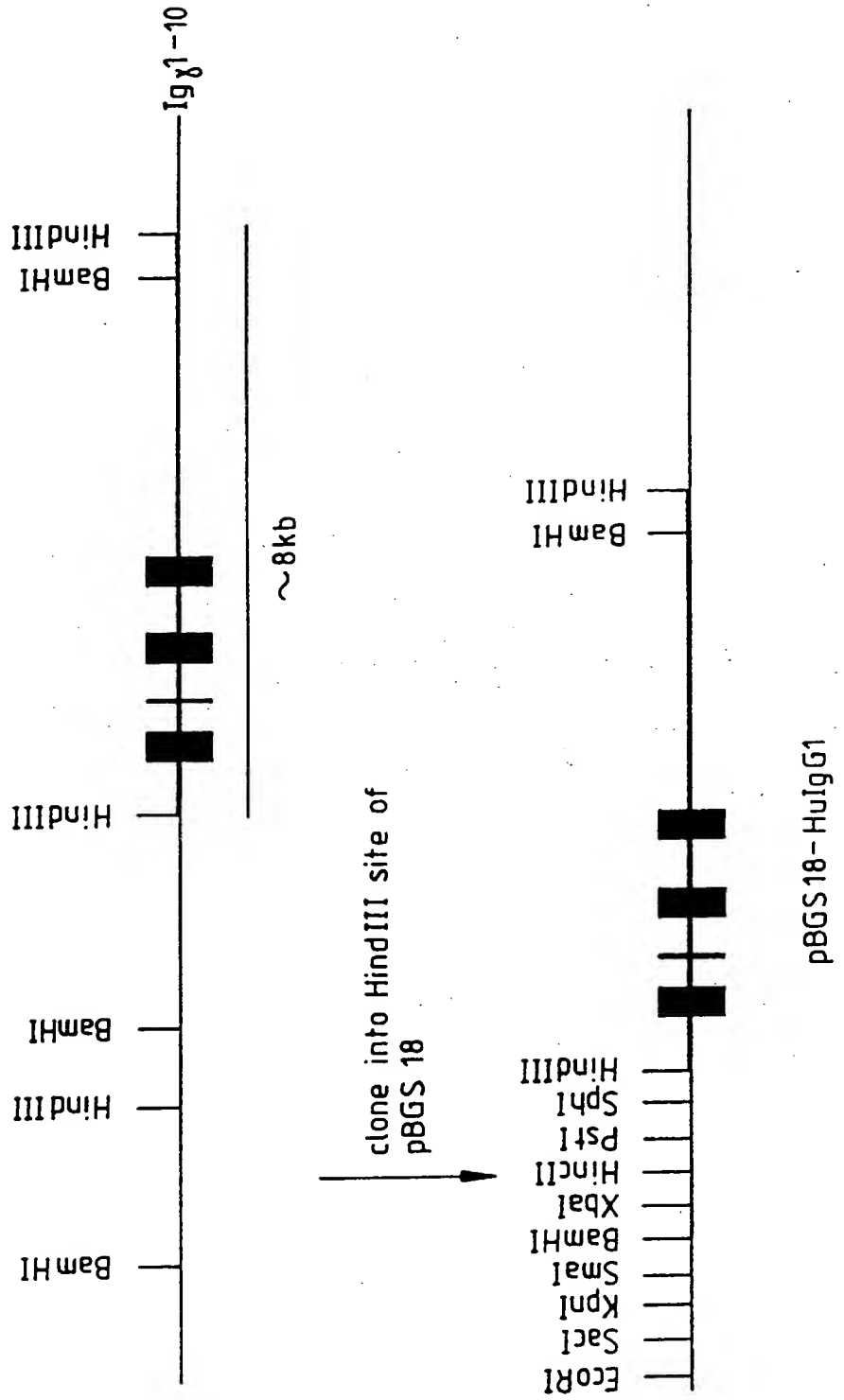
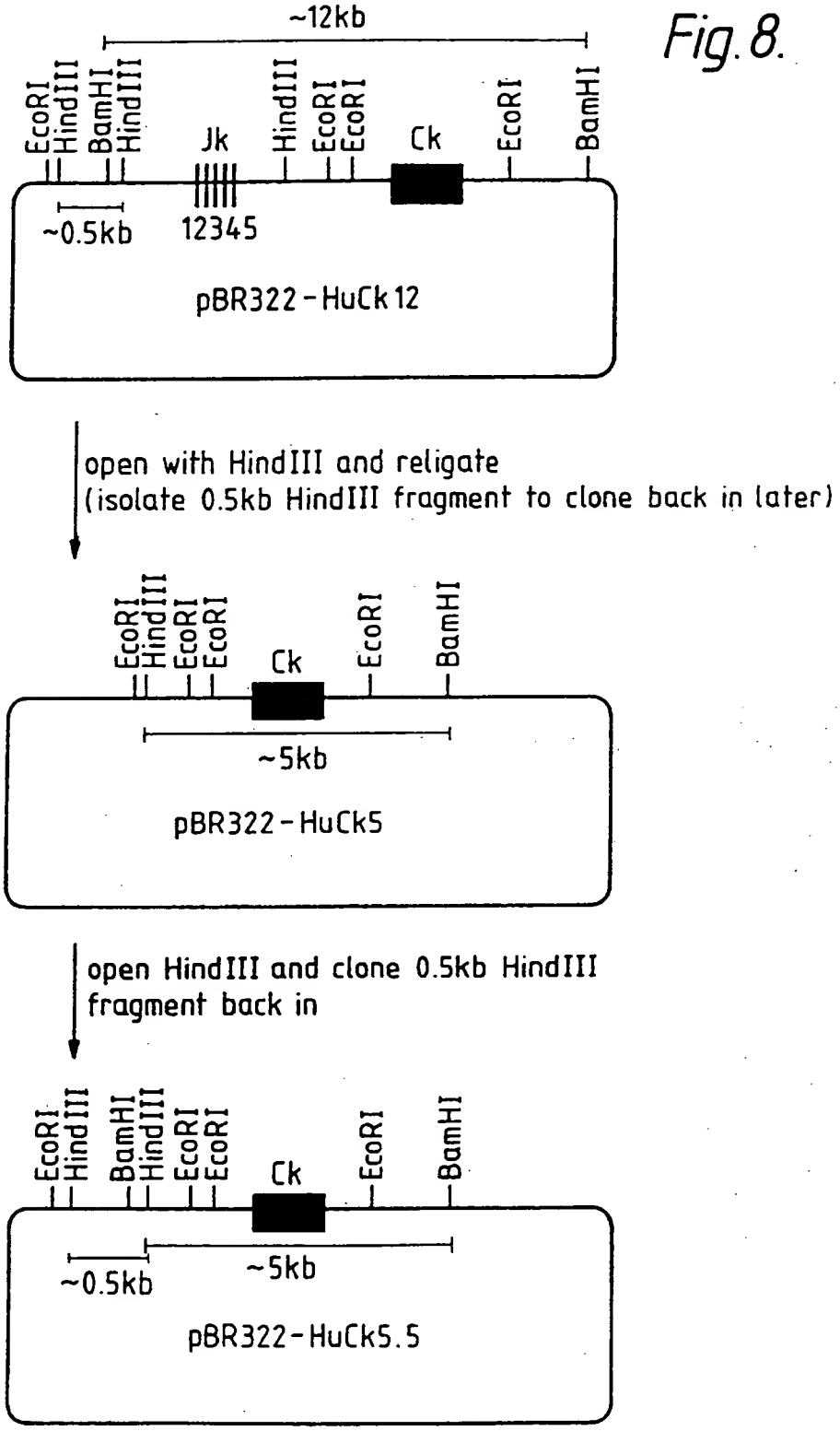

Igγ1-10
HindIII
BamHI
~8kb
HindIII
BamHI
HindIII
BamHI
clone into HindIII site of pBGS 18
HindIII
BamHI
HindIII
SphI
PstI
HincII
XbaI
BamHI
SmaI
KpnI
SacI
EcoRI
pBGS18-HuIgG1

Fig. 8.



Subclone HuCk containing BamHI fragment in pBGS18 - BamHI gives: pBGS18 - HuCk

Fig. 9.

Oligonucleotides used for cloning variable region genes.

I : mouse constant gamma1 primer

5' GAT AGA CAG ATG GGG GTG TCG TTT 3'

II : mouse constant kappa primer

5' AGA TGG ATA CAG TTG GTG CAG CAT 3'

Fig.10.

Oligonucleotides used to introduce KpnI and SalI in M13mp9HuVHLYS.

III : to introduce a KpnI in the HuVH leader intron

5' TGT CAT TGG TAC CCA TAT 3'

IV : to introduce a SalI 5' of the HuVHLYS gene

5' AAA TCT ATG TCG ACT GAA TAG 3'

Fig.11.

Oligonucleotides used for grafting of VkHMFG1 CDRs onto human kappa chain framework regions.

VI : VkHMFG1-CDR1

5' CTG CTG GTA CCA GGC CAA GTA GAT CTT TTG ATT GCT ACT ATA

TAA AAG GCT CTG ACT GGA CTT ACA GGT GAT GGT 3'

VII : VkHMFG1-CDR2

5' GCT TGG CAC ACC AGA TTC CCT AGT GGA TGC CCA GTA GAT

CAG CAG 3'

VIII : VkHMFG1-CDR3

5' CCC TTG GCC GAA CGT CCG AGG ATA TCT ATA ATA TTG CTG

GCA GTA GTA GGT 3'

Fig. 12.

HuVHIconHMFG1

```
                    5                  10                  15                  20
CAG GTG CAG CTG GTG CAG TCT GGG GCA GAG GTG AAA AAG CCT GGG GCC TCA GTG AAG GTG     60
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala Ser Val Lys Val

                   25                  30      CDR1        35                  40
TCC TGC AAG GCT TCT GGC TAC ACC TTC AGT GCC TAC TGG ATA GAG TGG GTG CGC CAG GCT    120
Ser Cys Lys Ala Ser Gly Tyr Thr Phe Ser Ala Tyr Trp Ile Glu Trp Val Arg Gln Ala

                   45                  50      52  A           55       CDR2
CCA GGA AAG GGC CTC GAG TGG GTC GGA GAG ATT TTA CCT GGA AGT AAT AAT TCT AGA TAC    180
Pro Gly Lys Gly Leu Glu Trp Val Gly Glu Ile Leu Pro Gly Ser Asn Asn Ser Arg Tyr

 60                      65                  70                  75
AAT GAG AAG TTC AAG GGC CGA GTG ACA GTC ACT AGA GAC ACA TCC ACA AAC ACA GCC TAC    240
Asn Glu Lys Phe Lys Gly Arg Val Thr Val Thr Arg Asp Thr Ser Thr Asn Thr Ala Tyr

 80      82  A   B   C           85                  90                  95
ATG GAG CTC AGC AGC CTG AGG TCT GAG GAC ACA GCC GTC TAT TAC TGT GCA AGA TCC TAC    300
Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Ser Tyr

 CDR3       100  A                  105                 110
GAC TTT GCC TGG TTT GCT TAC TGG GGC CAA GGG ACT CTG GTC ACA GTC TCC TCA            354
Asp Phe Ala Trp Phe Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
```

Fig. 13.

HuVkHMFG1

```
                    5                  10                  15                  20
GAC ATC CAG ATG ACC CAG AGC CCA AGC AGC CTG AGC GCC AGC GTG GGT GAC AGA GTG ACC   60
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr

                   25  27  A   B   C   D   E   F           30      CDR1
ATC ACC TGT AAG TCC AGT CAG AGC CTT TTA TAT AGT AGC AAT CAA AAG ATC TAC TTG GCC  120
Ile Thr Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser Ser Asn Gln Lys Ile Tyr Leu Ala

35                  40                  45                  50      CDR2
TGG TAC CAG CAG AAG CCA GGT AAG GCT CCA AAG CTG CTG ATC TAC TGG GCA TCC ACT AGG  180
Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr Arg

55                  60                  65                  70
GAA TCT GGT GTG CCA AGC AGA TTC AGC GGT AGC GGT AGC GGT ACC GAC TTC ACC TTC ACC  240
Glu Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr

75                  80                  85                  90      CDR3
ATC AGC AGC CTC CAG CCA GAG GAC ATC GCC ACC TAC TAC TGC CAG CAA TAT TAT AGA TAT  300
Ile Ser Ser Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Tyr Arg Tyr

95                  100                 105
CCT CGG ACG TTC GGC CAA GGG ACC AAG GTG GAA ATC AAA CGT                          342
Pro Arg Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
```

Fig. 14.

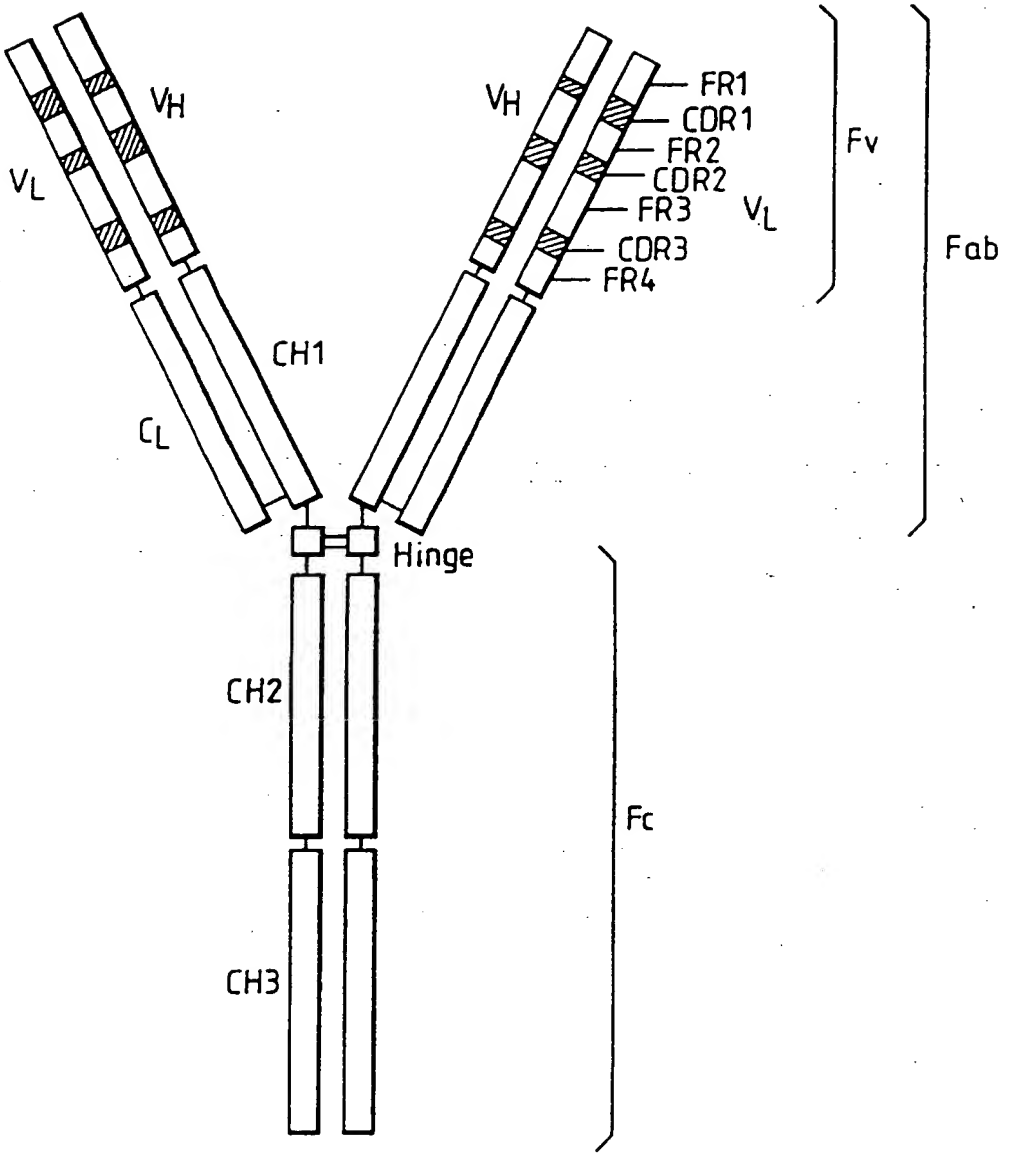
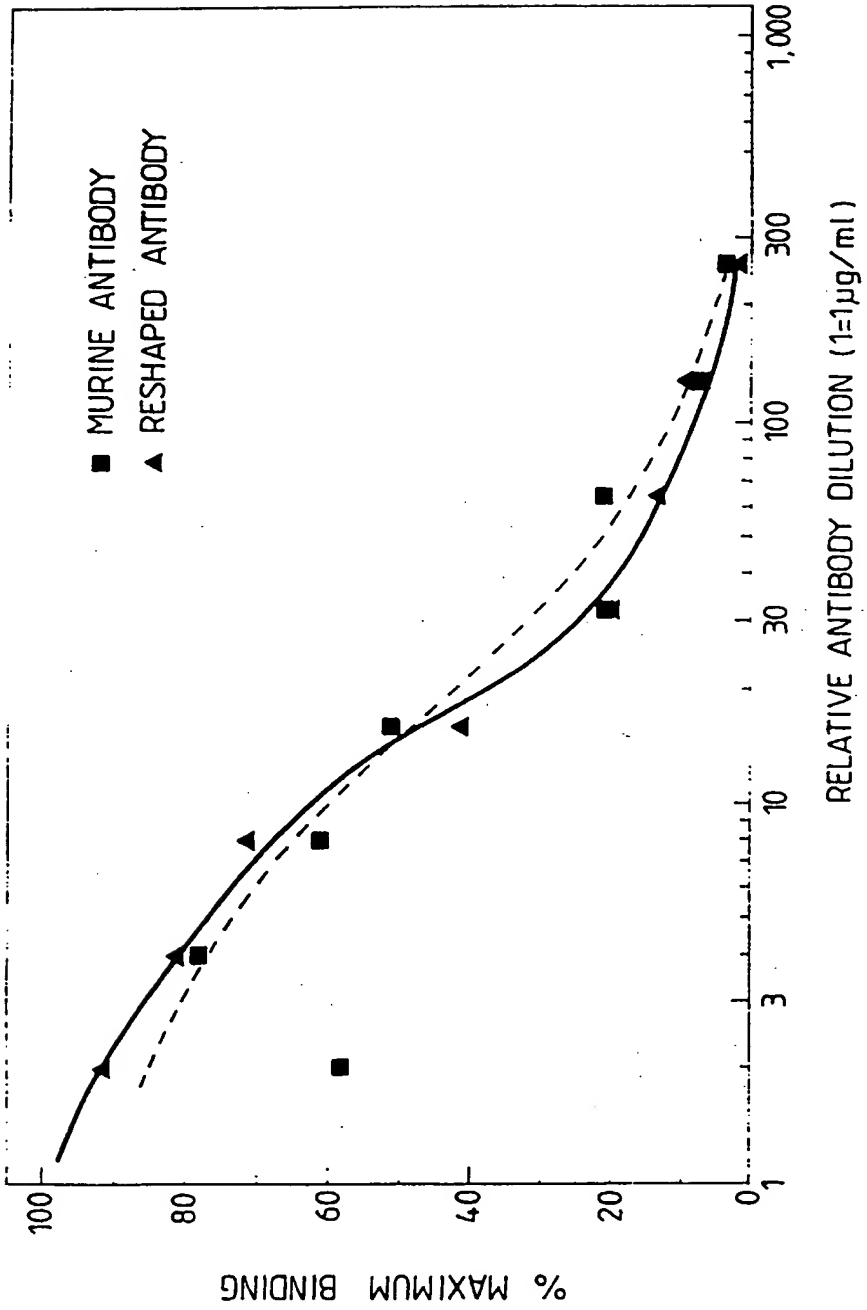

VH
VL
CH1
CL
VH
VL
FR1
CDR1
FR2
CDR2
FR3
CDR3
FR4
Fv
Fab
Hinge
CH2
CH3
Fc

Fig.15.


MURINE ANTIBODY
RESHAPED ANTIBODY
% MAXIMUM BINDING
0
20
40
60
80
100
1
3
10
30
100
300
1,000
RELATIVE ANTIBODY DILUTION (1=1µg/ml)

INTERNATIONAL SEARCH REPORT

International Applic PCT/GB 91/01511

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 07 K 15/28 C 12 P 21/08 C 12 N 1/21
C 12 N 15/13 A 61 K 39/395

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K C 12 P A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	NATURE, vol. 332, 24th March 1988, (London, GB), L. RIECHMANN et al.: "Reshaping human antibodies for therapy", pages 323-327, see page 325, right-hand column, line 5 - page 326, left-hand column, line 40 (cited in the application) ---	1-30
Y	EP,A,0369816 (THE UNIVERSITY OF MELBOURNE) 23 May 1990, see the whole document (cited in the application) ---	1-30
Y	WO,A,8907268 (JOHN MUIR CANCER & AGING INSTITUTE) 10 August 1989, see the whole document ---	1-30
Y	WO,A,9005142 (IMPERIAL CANCER RESEARCH TECHNOLOGY LTD) 17 May 1990, see claims 14-23 --- -/-	1-30

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
02-12-1991	21. 01. 92
International Searching Authority: EUROPEAN PATENT OFFICE	Signature of Authorized Officer: M.A.T. MORTHENSEN

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	WO,A,8809344 (CREATIVE BIOMOLECULES, INC.) 1 December 1988, see the claims	1-30
P,Y	WO,A,9107500 (UNILEVER PLC) 30 May 1991, see the whole document	1-30
P,Y	WO,A,9012319 (JOHN MUIR CANCER & AGING INSTITUTE) 18 October 1990, see the claims	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 30 is directed to a method of treatment of, and the diagnostic method practised on, the human body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. [ ] Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9101511 SA 51125

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 30/12/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0369816	23-05-90	CA-A-	2003211	17-05-90
WO-A- 8907268	10-08-89	AU-A-	3049189	25-08-89
		EP-A-	0401247	12-12-90
		JP-T-	3503120	18-07-91
WO-A- 9005142	17-05-90	EP-A-	0442926	28-08-91
WO-A- 8809344	01-12-88	AU-B-	612370	11-07-91
		AU-A-	1804988	21-12-88
		EP-A-	0318554	07-06-89
		JP-T-	2500329	08-02-90
WO-A- 9107500	30-05-91	AU-A-	6736990	13-06-91
		EP-A-	0429242	29-05-91
WO-A- 9012319	18-10-90	None		